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(57) Abstract

The present invention was made as a result of discovering an entire new approach and therapeutic target of cancer therapy. In addition, a prototype inhibitor compound, 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, has been discovered that acts by inhibiting cellular accumulation of specific PA species and is an inhibitor of PC directed PLDβ. Accordingly, the present invention provides a method for treating and preventing the spread of cancers comprising treating a patient with a tumor with an effective amount of a compound that reduces cellular accumulation of PA_4 species.

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USE OF COMPOUNDS WHICH INHIBIT PHOSPHATIDIC ACID FORMATION FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF CANCER

Technical Field of the Invention

The present invention provides a method for treating a wide variety of cancers using a novel approach to involve abnormal cell signaling mechanisms that are active in a transformed cellular phenotype. Specifically, the novel approach is based upon the discovery that certain phospholipid-based cell signaling enzymes are a common event of oncogenic mutations that result in abnormal and continuous stimulation of a phospholipid signaling pathway. Several inhibitors of this cell signaling mechanism are provided.

Background of the Invention

Cancer is a disease generally characterized by growth and spread of malignant tumors. Tumors result from rapid cell growth not controlled by the body's normal regulatory mechanisms. A tumor is considered malignant, and therefore a cancer, when it demonstrates unrestrained growth and a capacity to invade remote areas of the body. The exact causes of cancer are still largely unknown. However, recent discoveries have focused attention on a class of related genes that appear to control cancer cell growth and differentiation. These genes are termed "oncogenes" and are expressed in a variety of human cancers. When mutated, oncogenes encode for proteins, which appear to be involved in the process by which tumor cells respond to growth factors and other extracellular stimuli, and cause the cells to grow and proliferate in an unregulated "malignant" state. The malignant nature of cancer is its relentless progression throughout the body (a process termed metastasis), invading and destroying normal organs and leading to the death of the patient.

An important abnormal characteristic of cancer cells is their ability to metastasize (*i.e.*, breakthrough blood vessels and travel to distant sites in the body). Cancer cells secrete enzymes called metalloproteases which "breakdown" blood vessel walls and allow the cancer cells to enter the bloodstream and form tumors elsewhere. This process is termed proteolysis. One of these enzymes, called Type IV collagenase, is associated with tumors that metastasize or spread. Attachment of tumor cells to blood vessel walls and to normal organs occurs through tumor cell receptors (or adhesion receptors) called integrins. Attachment appears to be necessary to allow tumors to reside in different organs in the body.

Cancer is a disease generally characterized by the growth and spread of malignant tumors. Tumors result from rapid cell growth and are not controlled by the body's normal regulatory mechanisms. A tumor is considered malignant, and therefore a cancer, when it demonstrates unrestrained growth and a capacity to invade remote areas of the body.

An additional feature of cancer cells is their ability to secrete certain proteins, such as bFGF that stimulates the development of new blood vessels (a process termed angiogenesis or neovascularization). By doing so, as cancerous tumors enlarge, they develop new blood vessels to bring them a sufficient supply of nutrients for growth. The steps in tumor cell

growth and metastasis are shown in Figure 1. The 5 step cancer cascade includes all the components necessary for successful growth and spread (metastases) of tumor cells to distant sites: proliferation (*e.g.*, growth), proteolysis, migration, adhesion and angiogenesis.

More than 2 million cases of cancer were diagnosed in the U.S. in 1992. Among them, lung cancer, colon cancer and breast cancer constituted approximately 45% of the newly diagnosed cases. A specific oncogenic mutation, termed the *ras* oncogene, has been linked to the development of each of these types of cancers in more than 65% of cases.

Neovascularization is critical for the growth for tumors and is important in a variety of angiogenic diseases, such as diabetic retinopathy, arthritis, psoriasis and haemangiomas. More than 70% of cancer patients die from metastatic dissemination of the initial tumor. Tumor neovascularization is the crucial process for survival of a primary tumor and for metastatic dissemination. Angiostatic steroids and heparin with anti-angiogenic agents such as protamin have been used as therapies to suppress tumor growth. These therapeutic approaches have serious limitations, because when the dose of heparin exceeded an optimum level for inhibition of angiogenesis, both tumor growth and angiogenesis were stimulated. Also, high doses of cortisone that are required for antiangiogenesis led to immunosuppression. Acquisition of an angiogenic phenotype marked a transition from hyperplasia to neoplasia (Folkman et al., *Nature*, 339:58-60 1989).

Phosphatidic acid (PA) species have been shown by a number of investigators to act as intracellular second messengers produced following a variety of receptor-ligand interactions. Until recently, however, only a speculative understanding of the biologic relevance of this second messenger system was possible. Therefore, there is a need in the art to develop cancer therapies that affect all five steps and not just proliferation.

Different species of PA have different provenance and possibly different functions related to structure (Bursten et al., *J. Biol. Chem.* 266:20732-20743, 1991; Kanoh et al., *Cellular Signaling* 5:495-503, 1993; and Bursten et al., *Amer. J. Physiol.* 266:C1093-C1104, 1994). For example, the PA derived from phosphatidylinositol-directed phospholipase Cγ (PI-PLCγ), which is largely 1-stearoyl 2-arachidonoyl and 2-eicosatrienoyl PA (Bursten et al., *Amer. J. Physiol.* 266:C1093-C1104, 1994), is inhibitory to *ras*-deactivating protein, *ras*-GAP (Tsai et al., *Science* 243:522-426, 1989). In contrast, 1,2-sn-dipalmitoyl PA, which is derived largely from PC (Song et al., *Biochem. J.* 294:711-717, 1993), is not effective in inhibiting *ras*-GAP, but is effective in inhibiting *rho*-GAP (Tsai et al., *Mol. Cell. Biol.* 9:5260-5264, 1989).

PA's with varying provenance are produced simultaneously in response to the same stimulus. An example of this is the PDGF effect on Balb/3T3 fibroblasts. Kiss (Kiss, *Biochem. J.* 285:229-233, 1992) has demonstrated that PDGF activates a specific PE-directed phospholipase D (PE-PLD). Phospholipase D activity in this system, as well as PA mass, are associated with mitogenesis (Boarder, *Trends in Pharm. Sci.* 15, 57-62, 1994; and Boarder,

Trends in Pharm. Sci. 15, 57-62, 1994; and Fukami et al., *J. Biol. Chem.* 267:10988-10993, 1992). However, antibodies raised against PA generated during initial stimulation react primarily against 1,2-dilinoleoyl PA, but also strongly bind 1-stearoyl 2-arachidonoyl PA (Boarder, *Trends in Pharm. Sci.* 15, 57-62, 1994; and Fukami et al., *J. Biol. Chem.* 267:10988-10993, 1992). 1,2-dilinoleoyl PA production is a marker of activation of LPAAT (Kanoh et al., *Cellular Signaling* 5:495-503, 1993), whereas the latter PA species with arachidonate in the sn-2 position is associated with activation of PI-PLCγ and DG kinase.

Activation of phospholipase D does not always produce PA (or phosphatidylethanol in the presence of ethanol), but may produce either bis (diacylglycerol) phosphate (bis[PA]), bis (monoacylglycerol) phosphate (lyso(bis)PA), or closely-related species derived from phosphatidylglycerol. This is observed for a variety of glycerol forms containing free hydroxyl (-OH) moieties (Tettenborn et al., *Biochem. Biophys. Res. Comm.* 155:249-255, 1988; and Guillemain et al., *Amer. J. Physiol. 266:*C692-C699, 1994). Hence, lipid metabolism centering on PA and its relatives as a signaling molecule is apparently complex and structure-dependent.

Summary of the Invention

The present invention provides a method for treating cancer and preventing metastatic spread of cancer cells in a patient having cancer, comprising administering an effective amount of a compound that inhibits formation of phosphatidic acid (PA_4) species within cancer cells. Preferably the method for treating and preventing metastatic spread of cancer cells in a patient having cancer, comprises administering an effective amount of a compound that inhibits PC-PLDβ (phosphotidylcholine phospholipase D Type beta). Most preferably compounds that both inhibit formation of PA_4 species within cancer cells and inhibit PC-PLDβ activity include, for example, 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, and 1-(11-N-octylaminoundecyl)-3,7-dimethylxanthine.

Preferably the PA_4 species include, for example, PC-derived PA's having a myristylated and palmitoyl sn-1 and sn-2 side chains without alkyl, ether or vinyl ether side chains. Such PA_4's include, for example, those PA's seen in mass spectroscopy (Fab negative) having molecular weights of 619, 627, 643-649, 677, 703, 707 and 587. Most preferably, such PA_4 species include lyso (bis) PA species having arachidonate and polydocososaenate sn-2 and sn-1' species, and lyso (bis) PA sn-2 arachidonoyl and sn-2 eicosatrienoyl species.

The present invention provides a rational drug development approach to finding multiple-active compounds that are useful for the treatment of a wide variety of cancers, including, decreasing tumor cell growth by blocking oncogene induced events, blocking autocrine or paracrine growth factor stimulation of tumor cells, decreasing metastatic potential by blocking metalloprotease production, decreasing tumor adhesion to normal organs by blocking adhesion receptors, and decreasing the ability of tumors to induce nutrient carrying blood vessel formation by blocking bFGF or other tumor-dependent growth factor signaling.

Compounds with this spectrum of activities are useful for treating and preventing the metastatic growth of cancer cells and are illustrated herein. Such compounds (*i.e.*, small molecule pharmaceuticals) have prevented growth of a variety of human cancers in both laboratory and small animal models by regulating the activity of PC-PLDβ. Unlike traditional cancer chemotherapy, the illustrated compounds appear *in vitro* to be non-toxic to normal cells at concentrations which are lethal to cancer cells.

The present invention is further based upon the pioneering discovery that certain PA species are released in response to pro-inflammatory stimuli mediated by, by example, PDGF, EGF, FGF and VEGF, and that the increase in the PA species can be inhibited by addition of certain compounds. Therefore, this invention has resulted in the discovery of a new class of compounds useful for treating or preventing the progression of a large group of diseases mediated by such pro-inflammatory cytokines and treatable by inhibition of intracellular signaling of such pro-inflammatory cytokines. The data described herein shows that PA inhibition in response to inflammatory stimulus is useful for treating diseases associated with increased proliferation in response to PDGF, VEGF, EGF, or FGF or other heparin-binding growth factors such as Her2,3,4/regulin, IGF-1 or 2.

A number of intracellular signaling events take place following PDGF, EGF, FGF or VEGF binding to their respective cell surface receptors. All of the receptors in this class of cytokines possess intrinsic tyrosine phosphorylation activity. Shortly after binding, the receptors are phosphorylated at various sites in their intracellular domain by intrinsic tyrosine kinase activity of the receptor. This leads to the creation of additional binding sites for intracellular proteins. For instance, for PDGF these include phospholipase C-γ-1 (PLC-γ-1), the *ras* GTPase activating protein (GAP), phosphatidylinositol 3 kinase (PI3kinase), pp60c-*src*, p62c-*yes*, p50-*fyn*, *Nck*, and CRB2 as well as a 120 kd and a 64 kd species. Some of the proteins that associate with the receptor are signal transduction enzymes. For example, PLC-γ-1 is a specific phosphodiesterase that produces diacylgycerol (DAG) and inositol triphosphate, two second messengers that activate a serine/threonine specific protein kinase protein kinase C (PKC) and increase intracellular calcium levels. PI3kinase is a lipid kinase that phosphorylates the D3 position of phosphatidylinositol phosphatidylinositol-4-phosphate, or PI 4,5,P2. The physiological significance of this intracellular lipid species is unclear, but mutant PDGF receptors that no longer bind PI3kinase by virtue of a substitution of a specific tyrosine residue no longer proliferate in response to PDGF. In addition, PDGF induces activation of the serine/threonine kinase MAP kinase, via MAP kinase kinase, which may be activated by activation of *ras/raf* pathway. MAP kinase acts to activate the nuclear transcription factors c-*jun*, c-*fos* and possibly c-*myc*. PDGF, also up regulates increased transcription of these transcription factors.

Brief Description of the Drawings

Figure 1 illustrates a graph of illustrated PA species, including PA_1, PA_2, PA_3 and PA_4 species (note Fab negative mass spec. numbers) of NCI-H460 tumor cells before and after treatment with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (4 μM).

Figure 2 illustrates the effect of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine on spontaneous PA levels in two kinds of cancer cell lines. HT-29 and MCF-7 cells were treated with different concentrations of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine and the content of different PA determined by HPLC analysis. HT-29 colon carcinoma cells and MCF7 breast cancer cells were grown to 80% confluence, followed by fixation in ice-cold methanol, extraction of lipids, and separation of lipid species by HPLC. Under these conditions, each tumor cell line was found to spontaneously produce large amounts of differently migrating PA species, especially PA_1 species (migrating Rf 4-7) and PA_2 (Rf 9-11). The cells were grown in the presence of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at different concentrations, followed by a reexamination of lipids. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was found to suppress spontaneous production of these PA_1 and PA_2 species at the concentrations indicated.

Figures 3 and 4 show anti-proliferative (Figure 3) and anti-clonogenicity (Figure 4) of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine of HT-29 cells. HT-29 cells (1×10^5 cells/35 mm dish) were plated in McCoy's medium with 10% serum and incubated overnight. Concentrations of 3 and 6 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was added and viable cell counts made at the times shown. For clonogenic assays, treated and control cells (300/plate) were plated and allowed to grow colonies. After 7 days the colonies were fixed and counted. The values in Figure 4 are the means of 3 plates.

Figures 5 and 6 illustrate cytotoxicity and concentration dependence of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine against 3LL cells (Lewis lung carcinoma). 3LL cells (3×10^3 cells/well) were plated and incubated overnight in RPMI medium containing 10% serum. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was added at different concentrations and the cell number was determined at various time points by a vital dye uptake method. The values shown are triplicate of wells.

Figure 7 shows that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, even at much higher concentrations than shown having cytotoxicity to tumor cells, lacks cytotoxic activity in normal human bone marrow stromal cells. Human bone marrow stromal cells (1×10^4 cells/well) were plated in 96 well plates in McCoy's medium with serum and incubated overnight. Different dilutions of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine were added and viable cell counts made by vital dye uptake.

Figure 8 illustrates a densitometric analysis of matrix metalloproteinase expression measured from gelatin Zymogram gel with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-

dimethylxanthine. THP-1 human leukemia cells (1-2 x 10^6/35 mm dish) were plated in RPMI medium with 0.5% serum. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was added at 2.5 μM. Following 1 hour incubation, TNFα (0.5 ng/ml) was added and incubated for 18 hours. The supernatants from control and treated plates were collected and protease activity was determined in gelatin gels (zymogram) after electrophoretic separation of proteins.

Figures 9 and 10 show the effects of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine on matrigel invasion and viability in 3LL cells. 3LL cells (4.5 x 10^5 cells/well) were plated into the inner membrane of Matrigel chambers. Different concentrations of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine were added to the chamber and incubated for 48 hours at 37 °C. The cells on top of the membrane were removed and the cells that migrated to the bottom were stained with Diff Quick Solutions and scored for relative invasion. The effect of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine on viability of 3LL cells at different concentrations was determined separately.

Figure 11 illustrates the effect of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine on PDGF-BB (platelet-derived growth factor) induced proliferation of Balb/3T3 cells as a predictive assay of angiogenesis. 3T3 cells (3 x 10^3 cells/well were plated in DMEM with 10% serum in 96 well plates and incubated for 48 hours. Different dilutions of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine were added and incubated for 1 hour. PDGF (50 ng/ml) along with tritiated thymidine (1 μCi/ml) was added into each well. Cell proliferation was measured by measuring tritiated thymidine incorporation with each sample run in quadruplicate.

Figure 12 illustrates vascular endothelial growth factor (VEGF) induced proliferation of human umbilical vein endothelial cells (HUVEC) as an assay for adhesion. HUVECs were plated in EBM medium with serum and allowed to grow for 4 days. Different dilutions of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine were added to the plates, followed by VEGF (50 ng/ml) along with tritiated thymidine (1 μCi/ml). Proliferation was measured in quadruplicate and these data show the effect of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine to inhibit adhesion.

Figure 13 shows the effect of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine on THP-1 adherence to IL-1beta stimulated HUVECs. HUVECs (4 x 10^3 cells/well) were plated in RPMI medium with 10% serum and incubated for 48 hours. Different concentrations of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine were added and incubated for 1 hour. IL-1beta (15 ng/ml) was added and incubated for 6 hrs. Exponential growth THP-1 tumor cells, prestained with dye BCECF, were added (1.5 x 10^5 cells/well) and allowed to adhere for 20 min. The number of adhering tumor cells was determined after washing to remove non-adherent cells.

Figure 14 illustrates the effect of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine on VCAM-1 surface expression of TNFα stimulated HUVECs. This assay is a predictive model of adhesion. HUVECs grew to 90% confluence in 6 well plates in RPMI medium with 10% serum. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was added at different concentrations and incubated for 30 min. TNFα (20 ng/ml) was added and the cells were incubated for 5 hours. The cells were collected and the amount of VCAM-1 determined by indirect immunostaining followed by FACS (fluorescence activated cell sorter) analysis. Mean fluorescent intensity of TNF-stimulated cells was normalized to 100% with drug-treated samples expressed as a percent of control.

Figure 15 illustrates the effect of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine on ICAM-1 surface expression of TNFα stimulated HUVECs. This assay is a predictive model of adhesion. The procedures followed were the same as in Figure 14 except that the cells were stained with an ICAM-1 antibody.

Figure 16 illustrates the T cell and B cell response assays of the mice treated with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. Spleens from the treated mice were made into single cell suspensions in RPMI medium supplemented with 10% serum and placed (200,000 cells/well) in flat-bottomed 96 well plates. Anti-CD3 or a mixture of an anti-mu/IL-4 were added to the wells at final concentrations of 1 μg/ml and 1 μg/ml/12.5 ng/ml, respectively. Appropriate positive and negative controls were set up on each plate and all samples were assayed in quadruplicate. The plates were incubated for 2 days and proliferation was measured by tritiated thymidine incorporation.

Figure 17 illustrates an *in vivo* experiment showing that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine can arrest growth of Lewis Lung Carcinoma in mice. BDFI mice were injected (s.c.) with 1 x 10^6 3LL cells on day 0 and then treated with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (20 mg/kg i.p.), cyclophosphamide (20 mg/kg) or vehicle on alternate days beginning on day 7. The animals were sacrificed on day 20 and the lung tumors dissected and weighed. Figure 17 illustrates that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine showed superior results over an existing cancer chemotherapeutic agent.

Figure 18 illustrates platelet and neutrophil counts of the sacrificed mice in the experiment in Figure 17. In addition, platelet and neutrophil counts in the mice were not altered from vehicle, indicating that the bone marrow was not a side of toxicity for 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine.

Figure 19 illustrates mass spectroscopy tracings of HPLC-isolated PA and liso(bis)PA fractions from bone marrow stromal cells.

Figure 20 illustrates mass spectroscopy tracings of HPLC-isolated PA and liso(bis)PA fractions from NCI H460 tumor cells.

Figure 21 illustrates mass spectrometric (FAB-PI) analysis of HPLC-isolated PC fractions from NCI H460 tumor cells. HPLC-derived PC fractions from NCI H460 cells in the presence (21 hrs.) and absence of 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine were isolated, collected, dried under N_2, and stored at -70° C under argon. These fractions were then subjected to fast-atom bombardment mass spectrometry (FAB-MS) using a positively charged sulfuric acid/2-HEDS matrix, which caused the expulsion of positively-charged ions (FAB-PI).

Detailed Description of the Invention

The present invention was made as a result of discovering an entire new approach and therapeutic target of cancer therapy. In addition a prototype inhibitor compound, 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, has been discovered that acts by inhibiting cellular accumulation of specific PA species and was a likely inhibitor of PC directed PLDβ. Accordingly, the present invention provides a method for treating and preventing the spread of cancers comprising treating a patient with a tumor with an effective amount of a compound that reduces cellular accumulation of PA_4 species.

PA_1 species include LPAAT (lysophosphatidic acid acyl transferase)-derived PA's, such as dilinoleoyl (C18 unsaturated) PA's. PA_2 species include PE (phosphatidylethanolamine) directed PLD (phospholipase D) PA species, including alkyl, and alkeneyl (saturated) sn-1 and sn-2 PA species (*e.g.*, mass spectroscopy Fab negative 717, 737, 749, 773, 767 and 753). PA_3 species include PiG (glycan phosphatidylinostitol) directed PLD species, including 1-O- myristylated, myristylated, and myristylated palmitoyl PA species. PiGPLD is activated by the oncogene *v-src*. PA_4 species include PC (phosphotidylcholine) directed PLDβ including many myristylated PA species. According to the terminology employed herein, PC-PLDβ is distinguished from PC-PLDα. PC-PLDα is stimulated by sphingosine-1-phosphate, resulting in production of different PA species.

Investigation of these PA_4 inhibitors in *in vitro* and *in vivo* biologic systems has led to the following observations:

1. Inhibition of PA generation leads to inhibition of selective biologic effects transduced by specific receptor-ligand interactions.

2. Inhibition of PA does not inhibit normal homeostatic functions; high concentrations of these inhibitors are non-toxic to cells *in vitro* and have no apparent toxicities when given to animals at doses yielding plasma concentrations far in excess of their IC_{50}'s for biologic effects.

3. A series of compounds have been synthesized that inhibit several PA species that have layered biologic effects. For clarity we named these inhibitors Class 1 through Class 4 or PA_1 through PA_4 inhibitors.

The following Table 1 lists the designated PA species produced in response to receptor-ligand interactions and the respective class of inhibitor and prototypic compound.

Table 1

PA Class	Ligand or Stimulus	PA Species
1	IL-1 hypoxia	$PA_{1\alpha}$
2	PDGF FGF VEGF TNFα Type Ir (55kd)	$PA_{1\beta}$ PA_2
3	IL-2 IL-4 IL-7 TNFα type IIr (75kd)	PA_3
4	Mutated Oncogenes	PA_1 PA_2 PA_3 PA_4

4. In each case a compound selectively blocks one or more components of the biologic signal transduced by the ligand (*e.g.*, proliferation or upregulation of cell surface receptors) There is no apparent influence on other cellular processes which do not involve PA signaling. For example, activation of other signaling pathways, such as phosphorylation of receptors, or *src*-homologous proteins or activation of PLCγ are unaffected by compounds which inhibit PA production. Post receptor events such as MAP kinase activation, calcium flux, upregulation of nuclear protooncogenes, or proliferation signaled by ligands that do not use the PA pathway are similarly unaffected by these compounds.

PA_4 inhibitors, exemplified by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, have a broad spectrum of activities which indicate they have novel anti-cancer therapeutic properties. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was cytotoxic to all solid tumor cell lines tested with LC_{50}'s (the concentration at which 50% of cells die) of approximately 4 μM (micromolar) with an 8 to 18 hour exposure to the compound. At substantially higher concentrations 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine had no toxic effects on normal resting cells of various lineages. Its LC_{50} for fresh bone marrow myeloid progenitor cells (CFU-GM) was >27 μM with 8 hours of exposure, suggesting it had minimal bone marrow toxicity. The LC_{50} for non-tumor cells in log growth phase was more than 10-fold greater than that for tumor cells. Interestingly, after 3 months of testing, attempts to develop *in vitro* resistance have been unsuccessful, indicating that *in vivo* resistance may also be slow to develop.

Unlike current antineoplastic therapies, PA_4 inhibitors, exemplified by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, are unique in that they inhibit several major steps in tumor progression. Compounds of this class are cytotoxic and growth inhibitors to tumors. They also inhibit metastasis and angiogenesis. In addition to specific

cytotoxic effects, 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was a potent inhibitor of endothelial cell proliferation stimulated by PDGF, acidic or basic FGF and VEGF. The 50% inhibitory concentrations (IC_{50}'s) with each of these ligands is < 500 nM. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine also completely inhibited expression of the 92 kd, type IV collagenase (MMP9) associated with tumor invasion at a concentration of 2.5 μM by direct assays of gelatin gels. The functional effect of inhibition of collagenase is evidenced by the ability of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine to suppress tumor invasion into matrigel at concentrations of < 0.5 μM. Lastly, *in vitro* 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine inhibited adhesion of tumor cells to activated endothelial cells, at least in part, by suppressing the expression of ICAM and VCAM. These data indicate that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine inhibits tumor cell adhesion, extravasation and the development of an independent blood supply, and thus decrease metastasis through non-cytotoxic mechanisms in addition to doing the same via direct cytotoxicity.

In vivo pharmacokinetic studies have demonstrated that it is possible to achieve plasma levels of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine in excess of the LC_{50} for tumor cells without overt toxicity. Pharmacokinetic analyses following a single intraperitoneal (i.p.) dose illustrate that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine had a high volume distribution (approximately 20 L/kg) and a half life (t1/2) of greater than 6 hours with approximate linear elimination kinetics. At a dose of 10 mg/kg, the Cmax was approximately 2 μM and at 20 mg/kg was extrapolated to be approximately 4 μM. When dosed at 20 mg/kg i.p. every other day for 14 days, there were no apparent adverse effects on clinical observation, and on gross examination there were no apparent organ toxicities at time of autopsy. No significant weight loss was observed in treated animals. In addition there was no suppression of the neutrophil count, platelet count or of bone marrow or splenic cellularity. In treated animals, T and B cell mediated immune responsiveness was intact. Dosing of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at 10 mg/kg per day for 14 days, or at 20 mg/kg every other day (QOD) for 7 doses, was sufficient to achieve a reduction in growth of metastatic foci of B16 melanoma and Lewis lung carcinoma by approximately 80%. The compound also inhibited the primary tumor growth of Lewis lung carcinoma by 45% when established tumors were treated with the compound at 20 mg/kg QOD for 10 days, while cyclophosphamide at 20 mg/kg QD (a common cancer treatment agent) had no effect on the tumor growth. Cyclophosphamide treated animals had marked suppression in peripheral neutrophil and platelet counts, while 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine treated animals maintained peripheral blood counts similar to, or higher than, vehicle treated controls.

Table 2

Pharmacokinetic parameters for 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine

Dose (mg/kg)	Cmax (μM)	t1/2 (hrs)	AUC (μgohr/mL)	Cl (L/hr/kg)	Vd (L/kg)
10	*1.97	6.6	4.51	2.22	21.3
20	**3.44	11.9	10.31	1.94	33.2

* 30 minute peak concentration.

** First time point analyzed after i.p. administration of 20 mg/kg of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was one hour. Extrapolated 30 minute peak concentration is 3.44 μM.

In view of the lack of toxicity observed at the doses tested, and *in vitro* data suggesting that the maximal anti-tumor effects would be achieved if the plasma concentration were maintained at $\geq$ 4 μM for over 4 hours, a greater anti-tumor effect will be observed with optimal dosing. The animal data completely support the predictive *in vitro* observations, that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine or other PA_4 inhibitors are effective anti-tumor agents with minimal toxic effects on normal dividing cells in the bone marrow and the GI tract, the principal target organs for cancer chemotherapeutic side effects.

The following table shows solid tumor cell cytotoxicity of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine for different tumor cell lines. The data is expressed as the lethal concentration of drug wherein 50% of the cells have been killed by drug exposure

Table 3

	LC_{50} (μM)			
	4 hours	**6 hours**	**24 hours**	**72 hours**
Murine				
3LL (Lewis Lung)	9.76	3.40	4.80	3.36
Human				
HT-29 (colon)	5.61	5.62	4.12	3.26
NCI-H460 (large cell lung)	5.08	4.84	8.64	3.29
MCF-7 (breast)	<3.13	<3.13	7.56	2.25

Tumor cells were plated (3.5 x 10^3 cells/well) into individual wells of 96 well plates in complete medium with 10% fetal bovine serum. Cells were allowed to grow overnight at 37 °C in a 5% $C0_2$ incubator. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, at various concentrations, was added and incubated at 37 °C. At various time points the medium with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was replaced with fresh medium and incubated at 37 °C for 24 hours. The LC_{50} was determined based on the viable cell number as determined by BCECF, a vital

dye stain. Appropriate medium controls were set up, and all test samples and controls were run in triplicate.

Table 4

	LC_{50} (μM)		
	4 hours	**6 hours**	**24 hours**
Murine			
Wehi (AML)	4.46	2.34	2.19
P388 (monocytic leukemia)	18.9	7.62	5.76
HB9173 (myeloma)	9.01	6.96	3.26
Human			
Ramos (Burkitt's)	7.03	7.07	4.57
Raji (Burkitt's)	6.44	6.62	3.43
Jurkat (T-cell leukemia)	9.01	8.96	3.37
U937 (monocytic leukemia)	7.44	6.68	3.97
THP-1 (AML)	14.4	8.04	5.26
K562 (CML)	8.52	5.69	2.90
JM-1 (B-cell lymphoma)	ND	ND	8.36

Table 5 shows data from normal (not transformed) cells and their cytotoxicity to 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine

Table 5

	LC_{50} (μM)
Murine	
Mac-11 (IL-3 dep.)	>30
CFU-GM	>27
	LC_{50} (μM)
Human	
Bone marrow derived stromal cells	
confluent	>50
exponential	>50

Mac-11 (a murine IL-3 dependent cell line) and human bone marrow stromal cells were plated in 96 well plates in medium containing serum and various concentrations of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. After 4 hours incubation for Mac-11, or 24 hours for human stromal cells, the LC_{50} values were determined based on the number of viable cells in 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine exposed samples compared to controls as determined by a vital dye (BCECF).

Mouse bone marrow cells obtained from femurs were incubated with various concentrations of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine for 8 hours. Following incubation, the cells were washed and plated for colony forming unit-granulocyte

macrophage (CFU-GM) growth with spleen conditioned medium as the source of colony stimulating factor (CSF). After 7 days of incubation at 37 °C, the colonies formed were counted, and the LC_{50} was determined. Quadruplicate samples were set up for each compound dilution and control.

Drug resistance (using 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine) was determined. Approximately 2 x 10^7 *ras* transformed hamster 3T3 cells, or human NCI-H460 large cell lung tumor cells, were plated into medium containing 20 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. After 2 weeks the colonies from both groups were counted, and frequencies of resistance were found to be approximately 10^{-6}. Approximately 20 colonies were selected and propagated in the continuous presence of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at 20 μM. After 6 weeks of culture, the colonies continued to have slow doubling times (>72 hours), and incremental increases of the concentration of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine merely to 30 μM resulted in complete cell death.

Therefore, spontaneous drug resistance to 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine is a low frequency event (*i.e.*, 10^{-6}), thus attempts to develop resistant clones at incremental concentrations ("step wise selection") would prove near impossible. As a comparison, similar methods have been successfully employed in the past (Rice et al., *Proc. Natl. Acad Sci, USA* 83: 5978-5982, 1986 and Rice et al., *Proc. Natl. Acad. Sci, USA* 84: 9261-9264, 1987) using methotrexate and doxorubicin in generating drug resistant mutants with amplification of either the DHFR gene or the p-glycoprotein gene.

1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine HCl had a significant effect on PA and PA-related lipids in both marrow stromal cells and NCI H460 Tumor cells. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine caused an increase in PA and PA-related species containing particular acyl chains in marrow stromal cells. This reflects a diffuse increase in the endogenous species which are induced by growth in serum, which include arachidonoyl, arachidonoyl/docosapolyenoyl, and other long-acyl-chain, highly unsaturated PA and lyso(bis)PA species. Few linoleate-containing PA species are spontaneously synthesized in the marrow stromal cells. There is little evidence for suppression of serum-induced PA species by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine other than a slight effect on disaturated species, which represent a small fraction of serum-induced total PA/lyso(bis)PA mass. Lyso(bis)PA species containing arachidonate and other long acyl chains increase in short R_f PA fractions (R_f 6-10 min.), but later (longer R_f) fractions which also contain these lyso(bis)PA species show little significant change.

NCI H460 cells stimulated with serum demonstrate a more diffuse response in PA synthesis compared to marrow stromal cells. Not only arachidonoyl and long-acyl-chain docosapolyenoyl species were synthesized, but substantial concentrations of sn-2-linoleoyl and

alkyl\linoleoyl species were found. In addition, the disaturated species 1,2-dipalmitoyl, 1-myristoyl 2-stearoyl, and 1-palmitoyl 2-stearoyl PA were found to be increased after stimulation with serum. These latter PA species were present to a greater degree in NCI H460 cells as compared to marrow stromal cells. The advantages of mass spectrometric analysis of HPLC-peaks which migrate similarly is demonstrated by this finding.

1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine increased PA and lyso(bis)PA mass in NCI H460 cells. However, 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine abrogated an increase in linoleoyl, alkyl-linoleoyl, and disaturated PA species, while increasing arachidonoyl and heavy-chain docosapolyenoyl PA species. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine increased arachidonoyl-containing lyso-(bis)PA species in marrow stromal cells.

1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine increased PA and PA-related species which contain arachidonate and docosapolyenoates in all cells examined, but decreased the proportion of or abrogated PA species which contain linoleate, alkyl-linoleate, or are disaturated. The presence of a disaturated-acyl-specific PC-PLD is supported by data that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine had a marked effect on preventing hydrolysis of these disaturated sub-species of PC. The hydrolysis of disaturated PC species was inhibited by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, in agreement with the observations that disaturated PA species do not increase in the presence of the compound.

***In vivo* Studies**

B16 Melanoma

An *in vivo* study in mice was conductedwhere B16 melanoma cells were injected iv through a tail vein on day 0 and 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was administered i.p. at 10 mg/kg QD or 20 mg/kg QOC starting days 1-14. The mice were sacrificed on day 15 and the lungs were dissected and fixed in formalin. n addition bone marrow toxicity was evaluated by measuring neutrophil and platelet counts in the mice on day 15. These data are shown graphically below the lungs. These data show that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at either dose administered was not toxic to the bone marrow (in contrast to every existing cancer chemotherapy regimen known) and, in fact, increased counts over non-treated control animals.

The objective of this study was to demonstrate anti-metastatic activity of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine against B16 melanoma in an experimental metastasis model. B16 melanoma cells (1×10^5 per mouse) were inoculated intravenously (i.v.) into tail vein on day 0. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was administered i.p. at 10 mg/kg, once daily or 20 mg/kg, QOD starting day 1-14. The animals were sacrificed on day 15, and the lungs were dissected and fixed in formalin. The number of black metastatic foci were scored using a dissecting microscope.

Treated animals had a significant reduction in pulmonary metastasis. In addition, peripheral neutrophil and platelet counts at the end of treatment were substantially higher among 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine recipients than among vehicle treated controls. The data presented in Table 6 indicate that 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine inhibited the pulmonary metastasis in this model by 80% compared to controls.

Table 6

Effect of Repeated Dosing of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine on Pulmonary Metastasis of B16 Melanoma

	# PULMONARY FOCI			
Treatment Group (n=6)	**Mean ±SEM**	**Median**	**% Animals ≤ 50 Foci**	**p Value* (Wilcoxon)**
Vehicle	166 ±1 47	137	16.6%	-
10 mg/kg/day	46 ± 53	28	66.6%	0.025
20 mg/kg/QOD	67 ± 91	23	66.6%	0.025

* P value calculated versus vehicle control (Wilcoxon analysis)

Lewis Lung Carcinoma

The study objective was to demonstrate anti-tumor activity and lack of hematopoietic toxicity of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine in established tumors in mice. BDFI mice (from Charles River Laboratories) were injected subcutaneously with 1 x 10^6 cells on day 0. Animals were treated with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (20 mg/kg i.p.) on alternate days starting on day 7. The animals were sacrificed on day 20, and the tumors were dissected and weighed. Peripheral blood was also collected at autopsy, and the platelets and neutrophil counts were determined. A widely used anti-tumor agent, cyclophosphamide, was used as a positive control.

The data presented in Figures 17 through 18 demonstrate the treatment with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at 20 mg/kg QOD for 13 days resulted in 45% inhibition of primary tumor growth without any significant effect on peripheral blood counts, while cyclophosphamide at 20 mg/kg QD had no effect on the tumor growth.

In another *in vivo* study with 3LL cells, anti-tumor and anti-metastatic activity of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine in established pulmonary foci was established. 3LL cells (1 x 10^5 per mouse) were inoculated i.v. into the tail vein on day 0. 1-(11-Dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was administered i.p. at 10 mg/kg QD, or 20 mg/kg QOD, starting on day 7 after tumor implantation. Both dose groups were sacrificed on day 20, and the lungs were injected with black India ink to aid in the detection of tumor foci (white) against the normal lung tissue (black). Autopsied lungs with

tumors were shown depicting 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine's dramatic inhibition of tumor growth and metastasis of Lewis lung carcinoma.

Assay Procedures

Cytotoxicity Assay

Three thousand murine Lewis lung (3LL) cells were plated into individual wells of flat bottom 96 well plates in RPMI supplemented with 10% fetal bovine serum (FBS). Human bone marrow stromal cells were plated (10,000 cells per well) in McCoy's medium, supplemented with 12.5% FBS, 12.5% horse serum, 6% juice (1% pen.-strep., 1% glutamine, 1% vitamins, 0.8% essential amino acids, 1% sodium pyruvate, 1% sodium bicarbonate, 0.4% non-essential amino acids, 0.036% hydrocortisone) and fibroblast growth factor (FGF), at a final concentration of 10 ng/ml. Plates containing both cell lines were incubated overnight at 37 °C. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, at varying concentrations, was added to wells containing cells, and plates were incubated for various time points (4, 6 and 24 hours) at 37 °C. Appropriate medium controls were set up on each plate, and all test samples and controls were run in triplicate. Following incubation, the supernatant was removed and replaced with fresh appropriate growth medium then incubated overnight. BCECF [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester], diluted in phosphate buffer solution (PBS), was added at 10 μg/ml, and plates were incubated for 30 minutes at 37 °C. BCECF dye was removed from each well and replaced with PBS. Plates were read at 530 nm on a Millipore fluorescence plate reader. BCECF fluorescence has been shown to correlate linearly with viable cell numbers in the range of 500 - 50,000 cells per well. Average and standard deviations were calculated from BCECF fluorescence units of each well.

Clonogenic Assay

One hundred thousand human colon carcinoma (HT-29) cells were plated into individual wells of 6 well plates in McCoy's 5A supplemented with 10% FBS and incubated overnight at 37 °C. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, diluted in a solution made up of 50% PBS and 50% distilled H_2O, was added to the wells at a final concentrations of 6 μM and 3 μM. Samples were incubated for various time points (t=8 hour, 24 hour, 48 hour, 72 hour). Following each incubation, the samples were harvested by first collecting the cell supernatant from each well, then the cells were removed by trypsin. Each well treated with trypsin was added back to the corresponding supernatant sample and the cells were spun down. Each sample was re-suspended in 1 ml McCoy's 5A+10% FBS, and both live and dead cells were counted by trypan blue exclusion procedures. The cell suspensions of each sample are saved and replated at 300 cells per well of a 35 mm dish. The 35 mm dish samples were incubated for 7 days at 37 °C to allow for colony formation. Following this incubation the media was removed and the colonies were fixed with 10% formaldehyde for 1 - 2 hours. The formaldehyde was removed, and the colonies were stained

with 0.1% crystal violet stain for 30 minutes. The plates were washed multiple times with tap water and allowed to dry. Once dry, colonies of 20 or more cells were counted under a light microscope.

Matrigel Assay

Using 6 well Matrigel Invasion chambers, the 8μM inner membrane filter insert was rehydrated with assay medium consisting of RPMI supplemented with 0.1% bovine serum albumin (BSA). 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at varying concentrations, along with 450,000 murine Lewis lung (3LL) cells, both diluted in RPMI + 0.1% BSA, were added to the inner chamber on top of the membrane filter. The inserts containing cells, with and without 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, were placed in the plate which has wells also containing medium with and without 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. The Matrigel Invasion chambers, consisting of the inner membrane filter and the outer wells, were incubated for 48 hours at 37 °C. After incubation for 2 days, the cells on top of the filter that had not migrated through the inner membrane were removed using a cotton swab and washing the filter with medium. The filter membranes were stained with all three Diff Quick solutions, rinsed and allowed to air dry. Each membrane was removed from the inner chamber and mounted on slides (right side up). Photographs represent the field of each slide, showing those cells that have migrated through the inner membrane and adhered to the surface of the outer membrane.

CFU-GM Assay

Femoral cells were removed using a syringe containing RPMI supplemented with 10% FBS and a 20 gauge needle. Cell were counted, and marrow cellularity was calculated per femur. For the *in vivo* studies, the cells were plated, colonies assessed 7 days later and CFU-GM per femur calculated. In the *in vitro* studies, the cells were incubated at one million cells per ml in the presence or absence of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine and Vinblastine for 8 hours. Following incubation the cells were spun down and the supernatant discarded. The cells were washed extensively with RPMI + 10% FBS and re-suspended in 1 ml RPMI. Cell counts were performed, and cells were added to a Colony Forming Unit-Granulocyte Macrophage (CFU-GM) Assay (Stem Cell Technologies, Vancouver BC) at 50,000 cells per plate. Quadruplicate samples were set up for each sample dilution and control. After 7 days of incubation at 37 °C, the colonies were counted.

T-Cell and B-Cell Assays

Spleens were obtained from mice treated with and without 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, and a single cell suspension was prepared in RPMI medium supplemented with 10% FBS. Two hundred thousand cells were plated into individual wells of flat bottom 96 well plates. Anti-CD3, or a mixture of a Anti-mu/IL-4, were added to the appropriate wells at a final concentration of 1 μg/ml and 10 μg/ml/12.5 ng/ml

respectively. Appropriate positive and negative controls were set up on each plate, and all samples, along with the controls, were set in quadruplicate. The plates were incubated for two days at 37 °C. On day 2 the wells were pulsed with 1 μCi of tritiated thymidine (^{3}H-TdR), and the plates were incubated for an additional four hours. The plates were harvested, and the incorporation of ^{3}H-TdR was determined in a liquid scintillation counter. Average and standard deviations were calculated from the counts per minute (CPM) of each well.

TNF and IL-1 Induced Adhesion Assay

Two days prior to the adhesion experiment, 4,000 human umbilical vein endothelial cells HUVECs were plated into individual wells of round bottom 96 well plates in RPMI media supplemented with 10% FBS. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at varying concentrations was added to wells containing cells and plates were incubated for 1 hour at 37 °C. Following this pre-incubation, HUVECs were stimulated with human TNF alpha or IL-1 beta at a final concentration of 20 ng/ml and 15 ng/ml respectively. The plates were then incubated for 6 hours at 37 °C. Human monocytic leukemia cells (THP-1 cells) maintained in exponential growth were prestained with BCECF dye for 15 minutes at 37 °C. One hundred fifty thousand THP-1's, in RPMI supplemented with 1% FBS, were added to each well containing HUVECs, and the cells were allowed to adhere for 20 minutes at 37 °C. The plates were inverted and spun at 850 rpm to remove any additional cells. The plates were washed once with warm PBS to remove any additional non-adherent cells from the sides of the wells. PBS was added to each well and the plates were read at 485 nm/530 nm on a Millipore fluorescence plate reader. Average and standard deviations were calculated from the BCECF fluorescence units of each well.

VEGF Proliferation Assay

Four days prior to the proliferation assay, HUVECs were plated into individual wells of flat bottom 96 well plates in Endothelial Basal Media (EBM) supplemented with 5% FBS and endothelial growth factors. After this initial incubation at 37 °C, the media was removed and each well was washed once with PBS. EBM without growth factors and containing 0.5% fetal bovine serum, was added to wells for a 24 hour rest period. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at varying concentrations was added to wells containing cells, and plates were incubated for 1 hour at 37 °C. Following this pre-incubation, VEGF, was added to the wells at a final concentration of 50 ng/ml, along with 1 μCi of tritiated thymidine (^{3}H-TdR), and incubated for 18 - 24 hours. Appropriate positive and negative controls were set up on each plate, and all test samples and controls were ran in quadruplicate. The plates were then harvested, and the incorporation of ^{3}H-TdR was determined in a liquid scintillation counter. Average and standard deviations were calculated from the CPM of each well.

PDGF-BB Balb/3T3 Proliferation Assay

Murine Balb/3T3 cells were maintained in exponential growth. Cells were removed from flasks with EDTA. Three thousand cells were plated into individual wells of flat bottom 96 well plates in DMEM supplemented with 10% fetal bovine serum. After incubating for 48 hours at 37 °C the media was removed, and each well was washed once with PBS. DMEM containing 0.2% FBS was added to wells for a 24 hour rest period. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at varying concentrations was added to wells containing cells, and plates were incubated for 1 hour at 37 °C. Following this pre-incubation, PDGF-BB, was added to the wells at a final concentration of 50 ng/ml, along with 1 μCi of ^{3}H-TdR, and incubated for 18 - 24 hours. Appropriate positive and negative controls were set up on each plate, and all test samples and controls were run in quadruplicate. The plates were then harvested, and the incorporation of ^{3}H-TdR was determined in a liquid scintillation counter. Average and standard deviations were calculated from the CPM of each well.

Zymogram MMP Assay

THP-1 cells were plated at 1 - 2 million cells per well of a 6 well plate in RPMI medium supplemented with 0.5% bovine calf serum (CS). 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, diluted in RPMI + 0.5% CS, was added at a final concentration of 2.5 μM and incubated for 1 hour at 37°C. After this pre-incubation human TNFα, diluted in RPMI + 0.5% CS, was added to each well at a final concentration of 0.5 ng/ml. The plate was incubated overnight (approximately 18 hours). The following day, each well of cells and supernatant was harvested and spun down. One ml of supernatant sample was collected from each well. The remaining supernatant was removed and the cells were re-suspended in 0.5 ml RPMI + 0.5% CS. Each cell sample was counted using trypan blue exclusion, counting both live and dead cells. The supernatant samples were diluted 1:1 with 2X sample buffer, and each sample was loaded on to a Zymogram pre-poured gel from Novex. The Zymogram gel was run at 200 volts for approximately 1 hour. The gel was removed and incubated for 30 minutes at room temperature in Triton X-100. After Triton exchange, the gel was incubated overnight in a buffer containing 12.5 ml of 1.0 M Tris buffer pH 8.0, 2.5 ml 1.0M $CaCl_2$, 1.0 ml 0.5 mM $ZnSO_4$ and 500 ml H_2O. The following day the gel was fixed for 2 hours in a solution of 50% methanol, 10% glacial acetic acid and 40% H_2O. The gel was stained with coomassie blue at room temperature, for approximately 4 hours and de-stained overnight. A picture was taken of the gel, and the gel was analyzed using an image analyzer.

TNF and IL-1 Induced VCAM Surface Expression

Low passage, normal HUVECs were allowed to grow to 90% confluence in 6 well plates in RPMI medium supplemented with 10% FBS. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine was added, at varying concentrations, to wells containing cells and incubated for 30 minutes at 37°C. Following this pre-incubation, human TNFα was added at a final concentration of 20 ng/ml and incubated for 5 hours at 37 °C.

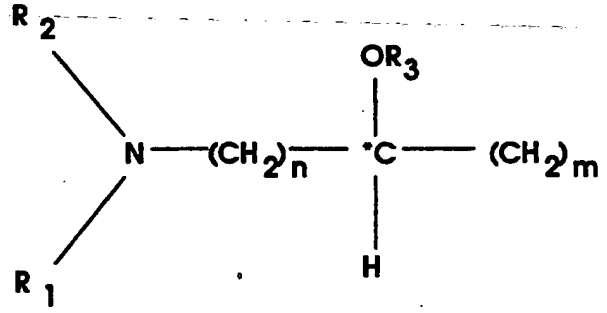
Cells were removed from each well by incubating 15 - 20 minutes with versene at 37 °C. Each well of cells was aliquoted into 5 ml polystyrene snap-cap tubes. Cells were spun down and washed twice with cold buffer consisting of PBS supplemented with 0.1% BSA and 0.01% Sodium Azide (Coulter EPICS Elite Fluorescene Activated Cell Sorter - FACS buffer). Keeping the tubes on ice, directly conjugated antibodies to Vascular Cell Adhesion Molecule-1 (VCAM-1), or the appropriate FITC-IgG control, was added to tubes at the manufacturer's recommended concentrations. Following incubations, each tube was washed twice with cold FACS buffer, and cells were resuspended in 500 µl FACS buffer. In addition, a 0.1 µg/ml concentration of propidium iodide was added to each tube for analysis of cell viability. Each tube was analyzed on the FACS with laser excitation at 488 nm and emission measured at 525 nm +/- 25 nm. Data were expressed as percent of control.

Compounds

PA_2 species are selected from the group consisting of 1-o-octadecanyl 2-oleoyl PA (687), 1-oleoyl 2-linoleoyl PA (697 or 698), 1-o-octadecanyl 2-linoleoyl PA (681), 1-o-octadecanyl-9,12-dienyl 2-linoleoyl PA (679), 1-myristoyl 2-oleoyl PA (645), 1-o-myristoyl 2-stearoyl PA (633), 1,2-sn-dilinoleoyl PA (695), 1-oleoyl 2-linoleoyl PA (697), 1-stearoyl 2-oleoyl PA (701), 1-o-oleoyl 2-20:4 PA (707), 1-o-linoleoyl 2-20:4 PA (705), 1-o-linoleoyl 2-20:5 PA (703), and combinations thereof. The numbers in parens next to each PA species shows the approximate molecular weight of the PA species as seen by mass spectroscopy analysis. More specifically, compounds comprise compounds and pharmaceutical compositions having the formula:

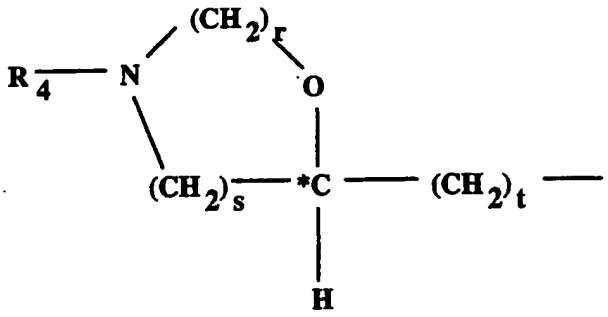
(X)j - (core moiety),

wherein j is an integer from one to three, the core moiety comprises at least one five to seven-membered ring or an open chain analog of such a ring group and X is a racemate mixture or R or S enantiomer of:



wherein *C is a chiral carbon atom; n is an integer from one to four; one or more carbon atoms of $(CH_2)_n$ may be substituted by a keto or hydroxy group; m is an integer from one to fourteen; independently, R_1 and R_2 are hydrogen, a straight or branched chain alkane or alkene of up to twelve carbon atoms in length, or $-(CH_2)_wR_5$, w being an integer from two

to fourteen and R_5 being a mono-, di- or tri-substituted or unsubstituted aryl group, substituents on R_5 being selected from the group consisting of hydroxy, chloro, fluoro, bromo, or C_{1-6} alkoxy; or jointly, R_1 and R_2 form a substituted or unsubstituted, saturated or unsaturated heterocyclic group having from four to eight carbon atoms, N being a hetero atom; and R_3 is hydrogen or C_{1-3}: or



wherein R_4 is a hydrogen, a straight or branched chain alkane or alkene of up to eight carbon atoms in length, $-(CH_2)_wR_5$, w being an integer from two to fourteen and R_5 being a mono-, di- or tri-substituted or unsubstituted aryl group, substituents on R_5 being selected from the group consisting of hydroxy, chloro, fluoro, bromo, or alkoxy C_{1-6} alkoxy, or a substituted or unsubstituted, saturated or unsaturated heterocyclic group having from four to eight carbon atoms, N being a hetero atom; r and s are independently integers from one to four; the sum (r + s) is not greater than five; t is an integer from one to fourteen; and one or more carbon atoms of $(CH_2)_s$ or $(CH_2)_t$ may be substituted by a keto or hydroxy group, or

X is independently a resolved enantiomer ω-1 secondary alcohol-substituted alkyl (C_{5-8}) substantially free of the other enantiomer, or X is a branched $-(CH_2)_a-CHR_6-(CH_2)_b-R_7$, wherein a is an integer from about 4 to about 12, b is an integer from 0 to 4, R_6 is an enantiomer (R or S) or racemic mixture (C_{1-6}) alkyl or alkenyl, and R_7 is a hydroxy, keto, cyano, chloro, iodo, flouro, or chloro group.

Preferably, the core moiety has from one to three, five to six-membered ring structures in a predominantly planar configuration. Preferably, the amino alcohol substituent (X) is bonded to a ring nitrogen if one exists. For example, the core moiety may be selected from the group consisting of substituted or unsubstituted barbituric acid; benzamide; benzene; cyclohexanedione; cyclopentanedione; delta-lactam; flutarimide; glutarimide; homophthalimide; imidazole amide; isocarbostyrile; lumazine; napthlalene; pthalimide; piperidine; pyridine; pyrimidine; pyrrole amide; quinazolinedione; quinazolinone; quinolone; recorsinol; succinimide; thymine; triazine; uracil or xanthine. Preferred cores include substituted or unsubstituted xanthine, more preferably halogen-substituted xanthine. Exemplary preferred cores include, but are not limited to: 1,3-cyclohexanedione, 1,3-cyclopentanedione; 1,3-dihydroxynaphthalene; 1-methyllumazine; methylbarbituric acid; 3,3-

dimethylflutarimide; 2-hydroxypyridine; methyldihydroxypyrazolopyrimidine (preferably, 1,3-dimethyldihydroxypyrazolo[4,3-d] pyrimidine); methylpyrrolopyrimidine (preferably, 1-methylpyrrolo [2,3-d] pyrimidine); 2-pyrrole amides; 3-pyrrole amides; 1,2,3,4-tetrahydroisoquinolone; 1-methyl-2,4(1H,3H)-quinazolinedione (1-methylbenzoyleneurea); quinazolin-4(3H)-one; alkyl-substituted (C_{1-6}) thymine; methylthymine; alkyl-substituted (C_{1-6}) uracil; 6-aminouracil; 1-methyl-5,6-dihydrouracil; 1-methyluracil; 5- and/or 6-position substituted uracils; 1,7-dimethylxanthine, 3,7-dimethylxanthine; 3-methylxanthine; 3-methyl-7-methylpivaloylxanthine; 8-amino-3-methylxanthine; and 7-methylhypoxanthine. Alternatively, the compound are a resolved R or S (preferably R) enantiomer of an ω-1 alcohol of a straight chain alkyl (C_{5-8}) substituted at the 1-position of 3,7-disubstituted xanthine. Preferably, the amino alcohol substituent (X) is bonded to a ring nitrogen if one exists. For example, the core moiety is selected from the group consisting of xanthine, halogen-substituted xanthines, 3,7-dimethylxanthine, 3-methylxanthine, 3-methyl-7-methylpivaloylxanthine, 8-amino-3-methylxanthine, 7-methylhypoxanthine, 1-methyluracil, 1-methylthymine, 1-methyl-5,6-dihydrouracil, glutarimides, phthalimide, 1-methyl-2,4(1H,3H)-quinazolinedione (1-methylbenzoyleneurea), 6-aminouracil, homophthalimide, succinimide, 1,3-cyclohexanedione, resorcinol, 1,3-dihydroxynaphthalene, 1,3-cyclopentanedione, 1,3-dimethyldihydroxypyrazolo[4,3-d] pyrimidine, 5-substituted uracils, 6- substituted uracils, 1-methylpyrrolo [2,3-d] pyrimidine, 1-methyllumazine, imidazole amides, 2-pyrrole amides, 3-pyrrole amides, benzamides, methylbarbituric acid, benzene, piperdine, delta-lactam, 2-hydroxypyridine, 1,2,3,4-tetrahydroisoquinolone, isocarbostyril, and quinazolin-4(3H)-one. Most preferably, the core moiety is a substituted xanthine, such as a 3,7 dimethylxanthine. The core moiety can also include a non-cyclic group. Examples of non-cyclic core groups include open chain analogs of glutarimide, carboxilic acid, a hydroxyl group, sulfone, sulfonate, and the like. Examples of compounds with demonstrated activity include compounds selected from the group consisting of R-1-(5-hydroxyhexyl)-3,7-dimethylxanthine, N-(11-octylamino-10-hydroxyundecyl)-homophthalimide, N-(11-octylamino-10-hydroxyundecyl)-3-methylxanthine, N-(11-octylamino-10-hydroxyundecyl)-2-piperdone, 3-(11-octylamino-10-hydroxyundecyl)-1-methyluracil, 3-(11-octylamino-10-hydroxyundecyl)-1-methyldihydrouracil, 1-(9-decylamino-8-hydroxynonyl)-3,7-dimethylxanthine, 1-(9-dodecylamino-8-hydroxynonyl)-3,7-dimethylxanthine, 1-(11-hexylamino-8-hydroxyundecyl)-3,7-dimethylxanthine, N-(11-phenylamino-10-hydroxundecyl)-3,7-dimethylxanthine, 1-(9-(2-hydroxydecyl-1-amino)nonyl)-3,7-dimethylxanthine, and combinations thereof.

Preferably, the core moiety is a member selected from the group consisting of substituted or unsubstituted: barbituric acid; benzamide; benzene; biphenyl; cyclohexanedione; cyclopentanedione; delta-lactam; flutarimide; glutarimide; homophthalimide; imidazole amide; isocarbostyrile; lumazine; napthlalene; pteridine; pthalimide; piperidine; pyridine; pyrimidine;

pyrrole amide; quinazolinedione; quinazolinone; quinolone; recorsinol; stilbene; succinimide; theobromine; thymine; triazine; tricyclododecane; uracil and xanthine.

Specific compounds are listed below.

Table 7

R-1-(5-hydroxyhexyl)-3,7-dimethylxanthine
N-(11-octylamino-10-hydroxyundecyl)-homophthalimide
N-(11-octylamino-10-hydroxyundecyl)-3-methylxanthine
N-(11-octylamino-10-hydroxyundecyl)-2-piperdone
3-(11-octylamino-10-hydroxyundecyl)-1-methyluracil
3-(11-octylamino-10-hydroxyundecyl)-1-methyldihydrouracil
1-(9-decylamino-8-hydroxynonyl)-3,7-dimethylxanthine
1-(9-dodecylamino-8-hydroxynonyl)-3,7-dimethylxanthine
1-(11-hexylamino-8-hydroxyundecyl)-3,7-dimethylxanthine
N-(11-phenylamino-10-hydroxundecyl)-3,7-dimethylxanthine
1-(9-(2-hydroxydecyl-1-amino)nonyl)-3,7-dimethylxanthine
N-(9-Octylamino-8-hydroxynonyl)phthalimide
N-(11-Octylamino-10-hydroxyundecyl)homophthalimide
1-(5-hydroxy-6-(N-benzyl)aminohexyl)-3-methylbenzoyleneurea
3-(11,10-Oxidoundecyl)quinazoline-4(3H)-one
N^2-(5-hydroxy-6-(N^3-propyl)aminohexyl)-(N^1-propyl)glutaramide
2-(11-Octylamino-10-hydroxyundeclcarboxamido)-octylcarboxamidobenzyl
1-Octylamino-2,11-undecadiol
1-(9-Octylamino-8-hdroxynonyl)-3-methylxanthine
1-(9-Tetradecylamino-8-hydroxynonyl)-3-methylxanthine
1-(11-Octylamino-10-hydroxyundecyl)-3-methylxanthine
7-(11-Octylamino-10-hydroxyundecyl)-1,3-dimethylxanthine
1-(11,10-Octylamino-10-hydroxyundecyl)-1-methyl-2,4-dioxotetrahydropteridine
1-(5-hydroxy-6-(N-benzyl)aminohexyl)-3,7-dimethylxanthine
1-(5-hydroxy-6-(N-propyl)aminohexyl)-3,7-dimethylxanthine
N-(11-Ocytlamino-10-hydroxyundecyl)glutarimide
N-(11-Octylamino-10-hydroxyundecyl)-2-piperidone
N-(11-Octylamino-10-hydroxyundecyl)succinimide
2-(11-Octylamino-10-hydroxyundecyl)-1,3-dimethoxybenzene
3-(5-hydroxy-6-(N-propyl)aminohexyl)-1-methyluracil
3-(9-Octylamino-8-hydroxynonyl)-1-methyluracil
3-(11-Octylamino-10-hydroxyundecyl)-1-methyluracil
3-(11-Octylamino-10-hydroxyundecyl)-1-methyldihydrouracil
3-(8-Octylamino-9-hydroxynonyl)-1-methylthymine

3-(5-hydroxy-6-(N-undecyl)aminohexyl)-1-methylthymine
3-(11-Octylamino-10-hydroxyundecyl)-1-methylthymine
3-(6-Propylamino-5-hydroxyhexyl)-1-methylthymine
1-(8-hydroxy-9-(N-benzyl)aminononyl)-3,7-dimethylxanthine
1-(5-hydroxy-6-(N-octyl)aminohexyl)-3,7-dimethylxanthine
1-(5-hydroxy-6-(N-(4-phenyl)butyl)aminohexyl)-3,7-dimethylxanthine
1-(6-Undecylamino-5-hydroxyhexyl)-3,7-dimethylxanthine
1-(5-hydroxy-6-(N-cyclohexylmethyl)aminohexyl)-3,7-dimethylxanthine
1-(5-hydroxy-6-(N-(6-hydroxy)hexyl)aminohexyl)-3,7-dimethylxanthine
1-(5-hydroxy-6-(N,N-dihexyl)aminohexyl)-3,7-dimethylxanthine
1-(5-hydroxy-6-(N-(4-methoxy)benzyl)aminohexyl)-3,7-dimethylxanthine
1-(8-hydroxy-9-(N-octyl)aminononyl)-3,7-dimethylxanthine
1-(5-hydroxy-6-(N-tetradecyl)aminohexyl)-3,7-dimethylxanthine
1[6-(Cyclopropylmethylamino)-5-hydroxyhexyl)]-3,7-dimethylxanthine
1-(6-Decylamino-5-hydroxyhexyl)-3,7-dimethylxanthine
1-(6-Dodecylamino-5-hydroxyhexyl)-3,7-dimethylxanthine
1-(11-Benzylamino-10-hydroxyundecyl-3,7-dimethylxanthine
1-(9-Decylamino-8-hydroxynonyl)-3,7-dimethylxanthine
1-(9-Tetradecylamino-8-hydrononyl)-3,7-dimethylxanthine
1-(9-Tetradecylamino-8-hydroxynonyl)-3,7-dimethylxanthine
1-(11-Hexylamino-10-hydroxyundecyl)-3,7-dimethylxanthine
1-(11-Octylamino-10-hydroxyundecyl-3,7-dimethylxanthine
1-(6-Allylamino-5-hydroxyhexyl)-3,7-dimethylxanthine
1-(11-Allylamino-10-hydroxyundecyl)-3,7-dimethylxanthine
1-(6-N-Methyloctadecylamino-5-hydroxyhexyl)-3,7-dimethylxanthine
1-(11-Decylamino-10-hydroxyundecyl)-3,7-dimethylxanthine
1-(11-Dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine
1-(11-Tetradecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine
1-[11-(4-Fluorobenzylamino)-10-hydroxyundecyl]-3,7-dimethylxanthine
1-[11-(4-Trifluoromethylbenzylamino)-10-hydroxyundecyl]3,7-dimethylxanthine
1-{11-(3-Diethylaminopropylamino)-10-hydroxyundecyl]-3,7-dimethylxanthine
N,N'-bis[(10-yl-9-hydroxydecyl)-3,7-dimethylxanthine]diaminododecane
1-(14-Bromo-13-hydroxytetradecyl)-3,7-dimethylxanthine
Aminobenzylamino)-10-hydroxyundecyl]-3,7-dimethylxanthine
1-[11-(3,4,5-Trimethoxybenzylamino)-10-hydroxyundecyl]-3,7-dimethylxanthine
1-[11-(3-Butoxypropylamino)10-hydroxyundecyl}3,7-dimethylxanthine
1-(14-Ocytlamino-13-hydroxytetradecyl]-3,7-dimethylxanthine
1-(11-Propylamino-10-hydroxyundecyl)-3,7-dimethylxanthine

1-(1-Undecylamino-10-hydroxydecyl-3,7-dimethylxanthine
1-(11-Phenylamino-10-hydroxyundecyl)-3,7-dimethylxanthine
N,N-bis[11-yl-10-hydroxyundecyl)-3,7-dimethylxanthine]undecylamine
1-(11-Octadecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine
1-[9-(N-Methyloctylamino-8-hydroxynonyl)]-3,7-dimethylxanthine
1-(4-Tetradecylamino-3-hydroxybutyl)-3,7-dimethylxanthine
1-[9-(2-hydroxydecyl-1-amino)nonyl)-3,7-dimethylxanthine
1-(6-Octadecylamino-5-hydroxyhexyl)-3,7-dimethylxanthine
1-[11-(N-Octylacetamido)10-hydroxyundecyl]-3,7-dimethylxanthine
11-Octylamino-10-hydroxyundecanoic acid amide
2-(11-Octylamino-10-hydroxyundecyl)-N-methylbenzamide
1-{11-(N-Methyl-N-octylamino)-10-hydroxyundecyl)-3,7-dimethylxanthine

Formulation and Dosage

It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables. A compound or a pharmaceutically acceptable salt or hydrate or solvate thereof is administered to a patient in an amount sufficient to be cytotoxic to tumor cells and to prevent metastatic spread and growth of cancer cells. The route of administration of the illustrated compound is not critical but is usually oral or parenteral, preferably oral. The term parenteral, as used herein, includes intravenous, intramuscular, subcutaneous, intranasal, intrarectal, transdermal, opthalmic, intravaginal or intraperitoneal administration. The subcutaneous and intramuscular forms of parenteral administration are generally preferred. The daily parenteral dosage regimen will preferably be from about 0.01 mg/kg to about 25 mg/kg of total body weight, most preferably from about 0.1 mg/kg to about 4 mg/kg. Preferably, each parenteral dosage unit will contain the active ingredient in an amount of from about 0.1 mg to about 400 mg. The compounds are generally active when given orally and can be formulated as liquids, for example, syrups, suspensions or emulsions, tablets, capsules and lozenges. A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s), for example, ethanol, glycerine, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavoring or coloring agent. A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose. A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule. Alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for

example, aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule. The daily oral dosage regimen will preferably be from about 0.01 mg/kg to about 40 mg/kg of total body weight. Preferably, each oral dosage unit will contain the active ingredient in an amount of from about 0.1 mg to about 1000 mg.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a compound or a pharmaceutically acceptable salt or hydrate or solvate thereof will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the particular patient being treated, and that such optimums can be determined by conventional techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment (*i.e.*, the number of doses of a compound or a pharmaceutically acceptable salt or hydrate or solvate thereof given per day and duration of therapy) can be ascertained by those skilled in the art using conventional course of treatment determination tests.

Example 1

This example illustrates HPLC-derived PA and PA-related fractions from marrow stromal cells in the presence and absence of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine were isolated, collected, dried under N_2, and stored at -70° C under argon. The lung cancer cell line NCI H460 and a non-transformed, primary marrow stromal cell line were used in these studies. The cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS). Before the FBS stimulation to initiate proliferation, the cells were starved in media containing only 0.5% FBS overnight. The compound tested, 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine at 10 μM was added 30 min. before the addition of the 20% FBS for stimulation. Cell stimulation was stopped by removal of media and the addition of cold methanol 18 hrs. after serum addition. Triplicate samples had the cells scraped from plates and transferred to stoppered glass centrifuge tubes. A volume of chloroform was added to complete the ratio of one volume aqueous material to 19 volumes of 2:1 chloroform:methanol.

Lipids in the samples were extracted by a modification of the procedure of Folch et al., 1957 , then separated and quantitated by normal phase HPLC using a gradient of hexane:2-propanol (3:4 v/v) and water in a gradient from 1 to 10% of the total volume and using ultraviolet detection at 217 and 206 nm. Fractions of the column effluent were analyzed by fast atom bombardment mass spectrometry (FAB-MS) for identification of sub-species within each lipid class.

FAB/MS spectra were acquired using a VG 70 SEQ tandem hybrid instrument of EBqQ geometry (VG Analytical, Altrincham, UK). The instrument was equipped with a standard unheated VG FAB ion source and a standard saddle-field gun (Ion Tech Ltd., Middlesex, UK) that produced a beam of xenon atoms at 8 KeV and 1 mA. The mass

spectrometer was adjusted to a resolving power of 1000 and spectra were obtained at 8 kV and at a scan speed of 10 sec/decade. 2-hydroxyethyl disulfide (2-HEDS) was used as matrix in the positive ion mode FAB/MS (FAB-PI), and triethanolamine was used as a matrix in the negative ion mode FAB/MS (FAB-NI). PA species observed in FAB-NI were native species coupled with lyso-PA breakdown M-H/z ions, and ranged between 550 and 820 mass units in FAB-NI. Because of differential protonation of the phosphate group of PA species in acidic target matrices, most PA species were detectable on FAB-PI as a larger range of ions, with most positive ions in clusters 2-4 mass units heavier than corresponding negative ions.

HPLC methods were capable of separating some lyso(bis)PA species from PA, and mass spectrometry of HPLC fractions allowed analysis of lyso(bis)PA sub-species. Other lyso(bis)PA species migrated with PA and were identified only by mass spectrometry. Lyso(bis)PA demonstrated a significant overlap with PA species in weights, as it did in charge distribution. The range of detected lyso(bis)PA species separated on this system began at 715-722 M-H/z and extended to hemi(bis) PA species (with 3 acyl groups, sn-1, sn-2, and sn-1' or 2') detected at 985-1100 M-H/z .

Semi-quantitative analyses using mass spectra were performed by ratios of selected ions found in repeated spectra compared to the matrix ions 231 and 307 (internal ratio 2.0-2.5). This was given by the ratio [selected ion]/ [231 + 307]. Comparing this generated ratio between similar mass spectra allowed confirmation of mass increases recorded by HPLC UV determinations. In addition, for mass spectrometric analyses where matrix ions were equivalent in intensity (*e.g.*, M/z 231+307 approximately equal to M/z 231+307 in separate determinations), indicating approximately similar total injected masses, internal ratios between peaks were computed to approximate changes in individual mass peaks. Table 8 identifies PA and lyso(bis)PA species in marrow stromal cells, and Table 9 identifies PA and lyso(bis)PA species in NCI H460 cells.

After serum stimulation for 21 hrs., there were increases visible in four separable PA or PA + lyso(bis)PA species: (1) R_f 6-7.5 min.; (2) R_f ~8-9.5 min.; (3) R_f 9.5-10 min.; and (4) R_f ~12.5-14. Each cell type appeared to have individualized increases or decreases in each of these HPLC-defined lipid fractions, both with serum and in the presence of 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. HPLC fractions were collected, solvent removed with N_2, and samples stored at -70° C under argon. Peaks of interest were analyzed using FAB-NI and FAB-PI mass spectrometry. Peaks were analyzed from the 21 hr. incubation time point for the marrow stromal and NCI H460 cells. Figure 19 illustrates fractions taken from the primary non-transformed human cell line, bone marrow stromal cells, after 21 hrs. of incubation with and without 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. Figures 19A-D represent marrow stromal cells in the presence of serum alone, Figure 19A'-D' represent marrow stromal cells in the presence of serum and 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. The mass spectrograms have

been normalized to each other, so that relative masses from analogous peaks (*e.g.*, Figures 19A and 28A') may be directly compared. Peaks are shown in descending order of total mass accumulation: Figures 19A/A' represent HPLC peak A5, B/B' peak A4, C/C' peak A2, and D/D' peak A7-13. In all mass spectrometric tracings illustrated, 567/569, 595/597/599, 603, and 615 are relatively constant ions, and regarded to be matrix ion/adducts and/or breakdown product adducts previously seen in PA fractions analyzed by FAB mass spectrometry.

Figure 19A shows significant mixed PA and lyso(bis)PA masses in peak A5 of marrow stromal cells stimulated with serum for 21 hrs. Peak A5 contained M-H/z 699 (1,2-dioleoyl PA), M-H/z 782 (1-eicosanoyl (C20:0), 2-docosatrienoyl (C22:3) PA; also this ion is consistent with 1-O-octadecanyl/1'-arachidonoyl lyso(bis)PA), and a cluster at M-H/z 808-812 (1-O-octadecanyl/1'-docosatrienoyl(C22:3), /1'-docosatetraenoyl(C22:4), and /1'-docosapentaenoyl (C22:5) lyso(bis)PA). M-H/z 855-858 (1-eicosanoyl, 1'-docosapentaenoyl (C22:5)/1'-docosatetraenoyl (C22:4) lyso(bis) PA) and M-H/z 883/884 (1-docosanoyl (22:0), 1'-docosapentaenoyl (C22:5) lyso(bis)PA) are present in lesser amounts. The presence of 10 µM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine had a profound effect on both PA and lyso(bis)PA species seen here (Figure 19A'). There was an increase in a variety of 1-O-tetradecanyl/oleoyl (myristyl) PA species (M-H/z 630,633), 1-O-tetradecanyl 2-arachidonoyl, 2-eicosapentaenoyl (M-H/z 651, 653), 1-O-hexadecanyl (palmityl) and palmitoyl/oleoyl PA species (M-H/z 660, 675), 1-O-octadecanyl (stearyl) 2-oleoyl PA species (M-H/z 687), 1-stearoyl 2-C20:3 and 2-arachidonoyl species (M-H/z 721,723), 1,2-diarachidonoyl PA (M-H/Z 742), and 1-O-eicosanoyl, 2-docosatetraenoyl (C22:4) PA (M-H/z 766), as well as >150% increases (normalized to matrix ions) in arachidonoyl- and docosapolyenoyl-containing lyso(bis)PA species at 808-810, 855, and 883. Largely missing from these mass spectrometric tracings are linoleate-containing PA species, alkyl-alkenyl PA species usually derived from PE, and myristoyl/palmitoyl species (contrast to Figures 20A, A'). The most likely identities of all detected mass spec. peaks for marrow stromal cells are given in Table 8.

The A4 peak also demonstrated similar features of interest. In Figure 19B, there were substantial amounts of palmitoyl/oleoyl, 1-O-hexadecanyl/oleoyl, and 1-O-tetradecanyl PA species, as well as 1-O-octadecanyl 2-stearoyl PA (M-H/z 689). Note that M-H/z 637 was an apparent matrix adduct of a breakdown product, and was found equally in both serum-treated and 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine-treated cells. There were shifts in mass, but little evidence for diminution in these peaks following incubation with 10 µM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (Figure 19B'). There were increases in representation in 1-stearoyl 2-C20:3 and 2-arachidonoyl PA's at 721-723 in Figure 19B', and equivalence in 1-eicosanoyl sn-2-C20-containing PA species between 740 and 770 M-H/z. In contrast, there was a diminution in two lyso(bis)PA species, M-H/z 799

(1-stearoyl, 1'-C20:3) and M-H/z 839 (1-o-docosanoyl, 1'-arachidonoyl) after treatment with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine.

Figure 19C shows mass spectrometric tracings from peak A2, in which 217 nm-determined mass decreased in the presence of 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. The best represented PA species include the previously observed M-H/z 630-631 (1-O-tetradecanyl 2-oleoyl PA) and M-H/z 673/675 (1-palmitoyl 2-oleoyl/2-stearoyl PA), with appearance of a new species, 647 (1,2-dipalmitoyl PA). These species diminish in response to 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, as seen in Figure 19C', correlating with a decrease in stimulated mass observed in this peak in marrow stromal cells. There is little evidence for an increase in mass in heavy chain (C20/C22) and polyunsaturated (> 2 double bonds) acyl PA, or lyso(bis)PA species in this fraction. Some of the explanation for a decrease in mass in this fraction, however, may be a shift of mass from one PA fraction (*e.g.*, R_f 5-6) to another (R_f 8-9.5), due to the presence of PA sub-species which associate with greater affinity with each other, or with lyso(bis)PA species.

Figure 19D shows the mass spec. data for peak A7-13 for marrow stromal cells. There was little change in either total mass observed or molecular species detected, both with serum (Figure 19D) and in the presence of 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (Figure 19D'). Lyso(bis)PA species were found in limited quantities except for M-H/z 885/886 (1-O-docosanoyl 1'-docosadienoyl (C22:4) lyso(bis)PA). Little qualitative change was found other than an increase in M-H/z 766 (1-palmitoleoyl/1'-arachidonoyl lyso(bis)PA) and a decrease in M-H/z 788 (1-O-eicosanoyl/1'-oleoyl). In summary, serum appears to stimulate a variety of (a) palmitoyl and stearoyl 2-polyunsaturated (n>2), (b) hexadecanyl- and tetradecanyl 2-oleoyl and 2-polyunsaturated, and (c) 1-saturated 2-arachidonoyl PA species in marrow stromal cells. There were also a variety of arachidonoyl and docosapolyenoyl lyso(bis)PA species that appeared with serum. These species were not inhibited by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, and arachidonoyl-PA and lyso(bis)PA species were stimulated by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine.

These fractions were then subjected to fast-atom bombardment mass spectrometry (FAB-MS) using negatively charged triethanolamine (TEM) matrices, which caused the expulsion of negatively-charged ions (FAB-NI). For PA, (bis)PA, and lyso(bis)PA, these represent almost invariably the native ions + lyso-PA breakdown products. These FAB-NI-detected ions from isolated PA and lyso(bis)PA fractions are illustrated in Figure 19, and the identity of each ion is detailed in Table 8 below.

Table 8

M-H/z	Identity
Peak A5 (Figure 1A)	
PA	

699	1,2-dioleoyl PA
782	1-eicosanoyl(C20:0) 2-docosatrienoyl (C22:3)
Lyso(bis)PA	
782	1-O-octadecanyl/1'-arachidonoyl
808-812	1-O-octadecanyl/1'-docosatrienoyl (C22:3)/ 1'-docosatetraenoyl (C22:4)/ 1'-docosapentaenoyl(C22:5)
855-858	1-eicosanoyl (C20:0)/1'-docosatetraenoyl 1'-docosapentaenoyl
	1-docosanoyl/1'-arachidonoyl
883-884	1-docosanoyl(C22:0)/1'-docosatrienoyl (C22:3)
Peak A5 + 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (Figure 19A')	
PA	
630	1-O-tetradecanyl 2-oleoyl
651-653	1-O-tetradecanyl 2-arachidonoyl/ 2-eicosapentaenoyl
660	1-O-hexadecanyl 2-oleoyl
673-675	1-palmitoyl 2-oleoyl/2-stearoyl
687	1-O-octadecanyl 2-oleoyl
698	1,2-dioleoyl
703	1,2-distearoyl
719,721,723	1-stearoyl 2-eicosatrienoyl (C20:3)/ 2-arachidonoyl/1-oleoyl 2-arachidonoyl
742	1,2-diarachidonoyl
751	1-eicosanoyl 2-eicosatrienoyl
766	1-O-eicosanoyl 2-docosatetraenoyl (C22:4)
Lyso(bis)PA	
803	1-oleoyl/1' eicosanoyl
808-812	1-O-octadecanyl/1'-docosapolyenoyl
853-855	1-eicosanoyl/1'-docosatetraenoyl 1'-docosapentaenoyl 1-docosanoyl/1'-arachidonoyl
883	1-docosanoyl/1'-docosatrienoyl
Peak A4 (Figure 19B)	
PA	
649	1-O-'en-tetradecanyl 2-eicosapentaenoyl
660	1-O-hexadecanyl 2-oleoyl
671-675	1-palmitoyl 2-linoleoyl/2-oleoyl
687-691	1-O-octadecanyl/2-stearoyl/2-oleoyl
707	1-O-octadecane-9-enyl/2-arachidonoyl
721	1-oleoyl 2-arachidonoyl/ 1-stearoyl 2-eicosatrienoyl
739	1-O-octadecanyl 2-docosadienoyl (C22:2)
747	1-stearoyl 2-docosahexaenoyl
766	1-O-eicosanoyl 2-docosatetraenoyl
Lyso(bis)PA	
799	1-stearoyl/1'-eicosatrienoyl (C20:3)
839	1-O-eicosanoyl/1'-docosatetraenoyl

863	1-O-docosanoyl/1'-docosahexaenoyl
899	1-O-tetracosanoyl/1'-docosadienoy

Peak A4 +1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (Figure 19B')

PA

649	1-O-'en-tetradecanyl 2-eicosapentaenoyl
675	1-palmitoyl 2-stearoyl
685	1-O-octadecane-9-enyl 2-oleoyl
689	1-O-octadecanyl 2-stearoyl
721	1-oleoyl 2-arachidonoyl
723	1-stearoyl 2-arachidonoyl
739	1-O-octadecanyl 2-docosadienoyl
756	1-docosanoyl 2-oleoyl

Lyso(bis)PA

773	1-oleoyl/1'-oleoyl
795	1-oleoyl/1'-arachidonoyl
835	1-O-eicosanoyl/1'-docosahexaenoyl
863	1-O-docosanoyl/1'-docosahexaenoyl

Peak A2 (Figure 19C)

PA

647	1,2-sn-dipalmitoyl
660	1-O-hexadecanyl 2-oleoyl
673-675	1-palmitoyl 2-oleoyl/1-palmitoyl 2-stearoyl
711	1-O-octadecanyl 2-eicosatrienoyl
755	1-docosanoyl 2-oleoyl/2-linoleoyl

Lyso(bis)PA

787	1-O-'en-octadecane-9-enyl/1'-eicosanoyl
831	1-eicosanoyl/1'-eicosanoyl
845	1-O-eicosanoyl/1'-docosanoyl

Peak A2 + 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (Figure 19C')

PA

647	1,2-sn-dipalmitoyl
660	1-O-hexadecanyl 2-oleoyl
673	1-palmitoyl 2-oleoyl
689	1-O-octadecanyl 2-stearoyl
721-723	1-stearoyl/1-oleoyl/2-arachidonoyl
767	1-O-eicosanoyl 2-docosatrienoyl

Peak A7-13 + 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine (Figures 19D, D')

Lyso(bis)PA

716	1-myristoyl/1'-linoleoyl
719	1-myristoyl/1'-oleoyl 1-palmitoyl/1'-palmitoleoyl
742	1-palmitoleoyl/1'-linoleoyl-
748	1-palmitoyl/1'-stearoyl
766	1-palmitoleoyl/1'-arachidonoyl
788	1-O-eicosanoyl/1'-oleoyl
818	1-oleoyl/1'-docosahexaenoyl

831	1-O-eicosanoyl/1-'docosahexaenoyl
855	1-eicosanoyl/1'-docosapentaenoy
	1-docosanoyl/1'-eicosatrienoyl
859-861	1-eicosanoyl/1'-docosatrienoyl/1'-docosadienoyl
885-886	1-O-docosanoyl/1' docosadienoyl
912	1-tetracosanoyl/1'-docosatrienoyl

Example 2

This example illustrates the HPLC and mass spectroscopy analysis performed in Example 1, except with lung NCI H260 tumor cells. NCI H460 cells were stimulated with serum in the presence and absence of 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. HPLC peaks were collected and analyzed by FAB-PI and FAB-NI mass spectrometry, and were normalized using adduct peaks M-H/z 567/569 and 615, and analyzed in descending order of absolute mass (with the greatest increase in mass analyzed first): Peak A4 in Figure 20A(i),A(ii), and A', peak A2 in Figure 20B/B', peak A5 in Figure 20C/C', and peak A7-13 in Figure 20D & E/D' & E'. Peaks A4 and A5 showed mass accretion with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, whereas peak 2 (which had significant total mass) and peak A 7-13 were essentially stable. Species identified by mass spec. analysis for NCI H460 cells are detailed in Table 9.

Figures 20A(i) and A(ii) illustrate the advantages of using mass spectrometry to analyze HPLC peaks, in combination with acyl chain analysis. The PA species content of this peak was in great contrast to the content of the A5 and A4 peaks from marrow stromal cells seen in Figures 19A,B/A,B'. Figures 20A(i) and A(ii) were normalized to 567/569, as was Figure 20A', which illustrates the contrasting subspecies masses. Figures 20A(i) and (ii) were both included because they demonstrate slightly different M-H/z peaks despite being taken from the same broad HPLC R_f area. The 21-hr. NCI H460 A4 peak demonstrated significant amounts of linoleoyl-containing PA species (M-H/z 671, 695, 697, 699, 705), as well as conspicuous amounts of 1,2-sn-dipalmitoyl PA/1-myristoyl 2-stearoyl PA (M-H/z 647). 1-stearoyl 2-arachidonoyl PA (M-H/z 723) was present, as are the longer acyl-chain species M-H/z 742, 749, and 766. Lyso(bis)PA species M-H/z 771 and 773 (1-oleoyl/1'-linoleoyl and 1-oleoyl/1'-oleoyl lyso(bis)PA) were also present, as was an alkyl C18/C22 cluster at M-H/z 808-810. Incubation of NCI H460 cells with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine resulted in significant suppression of M-H/z 647 and linoleoyl-containing species with the exception of M-H/z 671 (1-palmitoyl 2-linoleoyl PA) which may have different synthetic origins (*e.g.*, PC-PLD). In contrast, the primary sn-2 arachidonoyl PA, M-H/z 723 (1-stearoyl 2-arachidonoyl), was preserved in mass. A number of heavy chain lyso(bis)PA species, M-H/z 801, 849, and 879 were also present, with persistence of M-H/z 771 (in smaller amounts). A noteworthy feature of these lyso(bis)PA species is their marked linoleate content, which appeared to correlate with suppression of linoleoyl-containing PA

species. Although the UV-determined mass in HPLC peak A4 has increased, a substantial number of PA species containing linoleate have been suppressed. These effects were consistent throughout the NCI H460 peaks.

Mass spectrometric analysis of peak A2 (Figure 20B) revealed a similar profile of PA subspecies compared to peak A4, including M-H/z 647 (1,2-sn-dipalmitoyl PA) and 675 (1-palmitoyl 2-stearoyl PA), as well as 697, 699, and 707 [all linoleoyl-containing PA species, as in Peak A4 (Figure 20Ai-ii)]. Figure 20B' again revealed suppression of these specific species, with increase in lyso(bis)PA species containing linoleoyl and polyenoyl side chains (*e.g.*, M-H/z 801/831; M-H/z 897). Once again, both the highly saturated and linoleate-containing PA species were specifically suppressed, with reciprocal increase in arachidonoyl/polydocosaenoyl PA species. There was again a reciprocal increase in linoleate-containing lyso(bis)PA species.

HPLC peak A5, shown in Figure 20C, contained significant amounts of PA species with masses of M-H/z 647, M-H/z 695/697/699, M-H/z 721/723 (1-stearoyl, 2-20:3/2-arachidonoyl), M-H/z 739 (1-O-octadecanyl 2-docosadienoyl (C22:2)), M-H/z 742 (1,2-sn-diarachidonoyl), and M-H/z 754 (1-stearoyl 2-docosatrienoyl (C22:3)). Lyso(bis)PA species M-H/z 781 (1-O-hexadecanyl/1'-docosapentaenoyl), 798 (1-linolenoyl/1'-eicosanyl), 818-819 (1-oleoyl/1'-docosahexaenoyl (22:6)), and 827 (1-oleoyl/1'-docosadienoyl (C22:2)) were also present. Treatment of the NCI H460 cells with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine resulted again in a decrease of the disaturated and linoleate-containing PA species, with a small increase in 721/723, an increase in 1-oleoyl/1'-oleoyl lyso(bis)PA at M-H/z 773, and no change in lyso(bis)PA species at M-H/z 798 and 827. The lyso(bis)PA species M-H/z 853 (1-eicosanoyl/1'-docosatetraenoyl (C22:4) or 1-docosanoyl/1'-arachidonoyl lyso(bis)PA, especially given the significant 303 peak found in the linked mass scans: data not shown) had appeared.

Peak A7-13, which represented a lyso(bis)PA fraction, demonstrated stable mass in the presence of 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. Figures 20E and E' were expanded scale plots of Figures 20D and D', which show significant change in species content induced by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. Figures 20D and E illustrate a significant concentration of oleate and arachidonate-containing lyso(bis)PA species, which were confirmed by the linked scans of acyl chains with masses of 281 and 303 seen in Figure 20D. These species were consistent with those identified in marrow stromal cells in Figures 19D/D' and Table 8. Treatment with 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine resulted in a similar total mass, but significant shift in species content. Oleoyl and arachidonoyl species made a significant contribution to total mass, but in combination with stearoyl and linoleoyl lyso(bis)PA species. M-H/z 748 (1-stearoyl & 1-oleoyl/1'-palmitoyl), 774 (1-oleoyl/1'-oleoyl), 801/803 (1-linoleoyl & 1-oleoyl/1'-eicosanoyl), and 823/825 (1-linoleoyl/1' docosatrienoyl (22:3) & 1'-docosadienoyl (22:2)) lyso(bis)PA species were now present. This supports the hypothesis

that lyso-PA species, which accumulate due to inhibition of lyso-PA acyl transferase or transacylase enzymatic activities, are converted into lyso(bis)PA species. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine caused arachidonoyl and docosapolyenoyl acyl transfer into both PA and lyso(bis)PA. This was observed in both marrow stromal cells (Figures 19A'-D') and in NCI H460 cells. 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, in contrast, diminished synthesis of linoleoyl-containing PA, and caused accumulation of linoleoyl-containing lyso(bis)PA in tumor cell lines, but not in marrow stromal cells.

HPLC-derived PA and PA-related fractions from NCI H460 cells in the presence and absence of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine were isolated, collected, dried under N_2, and stored at -70° C under argon. These fractions were then subjected to fast-atom bombardment mass spectrometry (FAB-MS) using negatively charged triethanolamine (TEM) matrices, which caused the expulsion of negatively-charged ions (FAB-NI). For PA, (bis)PA, and lyso(bis)PA, these represent almost invariably the native ions + lyso-PA breakdown products. These FAB-NI-detected ions from isolated PA and lyso(bis)PA are illustrated in Figure 20, and the identity of each ion is detailed in Table 9 below.

Table 9

M-H/z	Identity
Peak A4	
PA	
645-647	1-myristoyl 2-oleoyl/2-stearoyl
	1,2-sn-dipalmitoyl
656	1-O-tetradecanyl 2-eicosatrienoyl
	1-O-hexadecanyl 2-linolenoyl
671	1-palmitoyl 2-linoleoyl
693-695	1-linoleoyl 2-linolenoyl
	1,2-sn-dilinoleoyl
699	1-stearoyl 2-linoleoyl
705	1-O-octadecane-9,12-dienyl 2-arachidonoyl
711-712	1--O-octadecanyl 2-eicosatrienoyl
723	1-stearoyl 2-arachidonoyl
745	1-oleoyl 2-docosahexaenoyl
749	1-stearoyl 2-docosapentaenoyl
Lyso-(bis)PA	
771	1-oleoyl/1'-linoleoyl
773	1-oleoyl/1'-oleoyl
793-795	1-linoleoyl/1-oleoyl/1'-arachidonoyl
810	1-O-octadecanyl/1'-docosapentaenoyl
852	1-eicosanoyl/1'-docosatetraenoyl
	1-docosanoyl/1'-arachidonoyl
885	1-docosanoyl/1'-docosadienoyl (C22:2)
920	1-tetracosanoyl/1'-docosanoyl (C22:0)

Peak A4 + 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine

PA	
667	1-myristoyl 2-arachidonoyl
671-673	1-palmitoyl 2-oleoyl/2-linoleoyl
689	1-O-octadecanyl 2-stearoyl
705	1-O-'en-octadecane-9,12-dienyl 2-arachidonoyl
721-725	1-oleoyl/1-stearoyl 2-arachidonoyl/2-eicosatrienoyl
Lyso-(bis)PA	
771	1-linoleoyl/1'-oleoyl
793	1-linoleoyl/1'-arachidonoyl
801	1-linoleoyl/1'-eicosanoyl
816	1-linoleoyl/1-linolenoyl/1'-docosahexaenoyl
849	1-eicosanoyl/1'-docosahexaenoyl
879	1-docosanoyl/1'-docosapentaenoyl (C22:5)
Peak A2	
PA	
647	1,2-sn-dipalmitoyl/1-myristoyl 2-stearoyl
673	1-palmitoyl 2-oleoyl
675	1-palmitoyl 2-stearoyl
691	1,2-sn-dilinolenoyl
695	1,2-sn-dilinoleoyl
707	1-O-octadecane-9,12-enyl 2-arachidonoyl
725	1-stearoyl 2-eicosatrienoyl
727	1-stearoyl 2-eicosadienoyl
745	1-oleoyl 2-docosahexaenoyl
749	1-stearoyl 2-docosapentaenoyl
767	1-eicosanoyl 2-docosapentaenoyl
Lyso-(bis)PA	
767	1-linoleoyl/ 1' linolenoyl
789	1-O-octadece-9-enyl/1'-eicosanoyl
803	1-oleoyl/1' eicosanoyl
810	1-O-octadecanyl/1-docosatetraenoyl
816	1-linoleoyl/1-docosahexaenoyl
867	1-docosapentaenoyl/1' docosahexaenoyl
884	1-docosanoyl/1'-docosatrienoyl
889	1-docosanoyl/1'-docosanoyl
901	1-docosaenoyl/1'O-tetracosanoyl

Peak A2 + 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine

PA	
647	1,2-sn-dipalmitoyl
649	1-O-'en-tetradecanyl 2-eicosapentaenoyl
660	1-O-hexadecanyl 2-oleoyl
687	1-O-octadecanyl 2-oleoyl
693	1-O-hexadecanyl 2-arachidonoyl
703	1,2-distearoyl
723	1-stearoyl 2-arachidonoyl
725	1-stearoyl 2-eicosatrienoyl
751	1-stearoyl 2-docosatetraenoyl

Lyso-(bis)-PA	
771	1-oleoyl/1'-linoleoyl
798	1-linolenoyl/1'-eicosanoyl
801	1-linoleoyl/1'-eicosanoyl
825	1-oleoyl/1'-docosatrienoyl
831	1-oleoyl/1'-docosanoyl
Peak A5	
PA	
647	1,2-sn-dipalmitoyl
	1-myristoyl 2-stearoyl
695	1,2-sn-dilinoleoyl
697	1-oleoyl 2-linoleoyl
721-723	1-stearoyl/1-oleoyl 2-arachidonoyl
	2-eicosatrienoyl (C20:3)
739	1-O-octadecanyl 2-docosadienoyl(C22:2)
742	1,2-sn-diarachidonoyl
745	1-oleoyl 2-docosahexaenoyl
754	1-stearoyl 2-docosatrienoyl
Lyso-(bis)PA	
781	1-O-hexadecanyl/1'-docosapentaenoyl
799	1-eicosanoyl/1'-linolenoyl
816	1-linoleoyl/1-linolenoyl/1'-arachidonoyl
827	1-oleoyl/1' docosadienoyl
831	1-oleoyl/1-docosanoyl
894-903	1-O-tetracosanyl/1'-docosanoyl
	1'-docosaenoyl, docosadienoyl, docosatrienoyl
929	1-O-tetracosanyl/1'-tetracosanyl
Peak A4 + 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine	
PA	
649	1-O-'en-tetradecanyl 2-eicosapentaenoyl
660	1-O-hexadecanyl 2-oleoyl
673	1-palmitoyl 2-oleoyl
693	1-linoleoyl 2-linolenoyl
723-725	1-stearoyl 2-eicosatrienoyl/2-arachidonoyl
742	1,2-sn-diarachidonoyl
749	1-stearoyl 2-docosapentaenoyl
756	1-stearoyl 2-docosadienoyl
Lyso-(bis)PA	
773	1-oleoyl/1'-oleoyl
781	1-O-hexadecanyl/1'-docosapentaenoyl
798	1-linolenoyl/1'-eicosanoyl
827-831	1-oleoyl/1'-docosadienoyl/1'-docosaenoyl
853	1-eicosanoyl/1'-docosatetraenoyl
	1-docosanoyl/1'-arachidonoyl
873	1-O-docosanoyl/1'-docosaenoyl
902	1-O-docosanoyl/1'-tetracosanoyl
Peak A7-13	
Lyso-(bis)PA	

717	1-myristoyl/1'-linoleoyl
743	1-palmitoyl/1'-linoleoyl
836	1-stearoyl/1'-docosanoyl
862	1-eicosanoyl/1'-docosanoyl
886	1-docosanoyl/1'-docosadienoyl

Peak A7-13 + 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine

Lyso-(bis)PA

718	1-myristoyl/1' linoleoyl/1'-oleoyl
748	1-stearoyl/1-oleoyl/1'-palmitoyl
774	1-oleoyl/1'-oleoyl
801	1-linoleoyl/1'-eicosanoyl
803	1-oleoyl/1'-eicosanoyl
823	1-linoleoyl/1'-docosatrienoyl
825	1-oleoyl/1'-docosatrienoyl
	1-linoleoyl/1'-docosadienoyl

Example 3

This example illustrates the mass spectrometric determination of changes in phosphatidylcholine (PC) species in the NCI H460 tumor cell line induced by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. A conspicuous finding in mass spectrometric analysis of PA fractions from NCI H460 cells was the presence of diunsaturated PA species, particularly M-H/z 647 (1,2-sn-dipalmitoyl and 1-myristoyl 2-stearoyl PA). These PA species were conspicuously suppressed by pre-incubation with 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. The commonest location for possible precursors of these PA species, particularly given the paucity of palmitoyl CoA acyltransferases or palmitoyl transacylases in the majority of cellular membranes, were PC species in the plasma membrane. PE fractions contain relatively few fully saturated species, and hence were less likely to serve as precursors for disaturated PA species. A phospholipase D activity specific for saturated acyl chains and directed against PC has been associated with the oncogene *v-src*. We examined PC species in NCI H460 cells in order to evaluate the possibility that these were being visibly affected by serum in tumor cells (*i.e.*, were having saturated PC species selectively hydrolyzed), and if this activity might be blocked by 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. For molecular species in which the individual ions are not changing due to external conditions, the ratio of ion M-H/z determinations of each sub-species was relatively constant. Where there are significant changes due to external factors, such as phospholipase D, these ratios may change significantly.

In Figure 21A, PC species (R_f 24-27 min.) from NCI H460 cells grown in serum for 21 hrs. are shown. Under these conditions, there was a change in internal ratio between PC sub-species observed. The ratios obtained are as follows (for given M+H/z species): 759-761/704-707 = ~25, 759-761/718-719 > 40, 759-761/735 = 10, and 759-761/787-789 = 1. M+H/z 704-707 represent 1-myristoyl, 2-palmitoyl/2-palmitoleoyl PC, 718-719 represents 1-O-

tetradecanyl 2-linoleoyl PC, 735 represents 1,2-sn-dipalmitoyl and 1-myristoyl 2-stearoyl PC, 759-761 represent 1-palmitoyl, 2-oleoyl/2-linoleoyl PC, and 787-789 represent 1-stearoyl, 2-stearoyl/2-oleoyl/2-linoleoyl PC. The presence of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine resulted in an increase in mass of ~15-20% in the PC fractions, and a change in the ratios (Figure 21B). The ratios obtained in this figure are: 759-761/704-707 = ~11.25, 759-761/718-719 = ~22.5, 759-761/735 = 2.3, and 759-761/787-789 = 3.0. The ratio of the dominant PC sub-species in NCI H460 cells had changed in the presence of 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine. The ratio difference, irrespective of mass, was observed by comparing Figure 21B to 21C, which showed the FAB-MS (PI) analysis of PC species from NCI H460 cells in the absence of serum; the 759-761/704-707 ratio = 5.6, the 759-761/718-719 ratio = 23, 759-761/735 = 2.0, and 759-761/787-789 = 3.2. These data show that growth of tumor cells in serum resulted in a diminution in total PC mass, and a relatively selective diminution in alkyl tetradecanyl/myristoyl/palmitoyl (704-717, 735, 759-761) species, as serum results not only in decrease in PC mass, but greater loss of the 704-707 and 735 species relative to 761 than the other PC species, or 761 relative to 787-789. These data correlated significantly with production in the tumor cells of the M-H/z species 647, 1,2-sn-dipalmitoyl and 1-myristoyl 2-stearoyl PA. As pre-incubation of NCI H460 cells with 10 μM 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine resulted in loss of these PA species in the tumor cells, and a parallel increase in the corresponding PC species. Therefore, PC-PLD activity directed against saturated PC species (related to or equivalent to that seen with *v-src* activation of PC-PLD) was inhibited by 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent.

We claim:

1. A method for treating or preventing metastatic spread of cancer cells in a patient having cancer, comprising administering an effective amount of a compound that inhibits formation of phosphatidic acid (PA_4) species within cancer cells.

2. The method of claim 1 wherein the compound further inhibits PC-PLDβ (phosphotidylcholine phospholipase D Type beta).

3. The method of claim 2 wherein the compound both inhibit formation of PA_4 species within cancer cells and inhibits PC-PLDβ activity

4. The method of claim 1 wherein the compound is 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine or 1-(11-N-octylaminoundecyl)-3,7-dimethylxanthine.

5. The method of claim 1 wherein the PA_4 species include PC-derived PA's having a myristylated and palmitoyl sn-1 and sn-2 side chains without alkyl, ether or vinyl ether side chains.

6. The method of claim 5 wherein the PA_4 species include those PA's seen in mass spectroscopy (Fab negative) having molecular weights of 619, 627, 643-649, 677, 703, 707 and 587.

7. A rational drug development method for discovering multiple-active cancer therapeutic compounds that are useful for the treatment of a wide variety of cancers, including, decreasing tumor cell growth by blocking oncogene induced events, decreasing metastatic potential by blocking metalloprotease production, decreasing tumor adhesion to normal organs by blocking adhesion receptors, and decreasing the ability of tumors to induce nutrient carrying blood vessel formation by blocking bFGF or other tumor-dependent growth factor signaling, comprising screening candidate drugs in cancer cells for inhibition of PA_4 species.

8. The method of claim 7 wherein assays for determining PA_4 species within cells is accomplished by an initial separation of phospholipids into a PA fraction by high performance liquid chromatography and a determination of PA species by mass spectroscopy.

9. A method for suppressing tumor growth via paracrine or autocrine mediated responses to PDGF, FGF EGF or VEGF that is useful for treating or preventing progression of tumors stimulated through overexpression of *her-2-neu* receptor, wherein the method

comprises administering a compound that inhibits signal transduction through cellular accumulation of non-arachidonoyl phosphatidic acid (PA) selected from the group consisting of 1-o-octadecanyl 2-oleoyl PA (687), 1-oleoyl 2-linoleoyl PA (697 or 698), 1-o-octadecanyl 2-linoleoyl PA (681), 1-o-octadecanyl-9,12-dienyl 2-linoleoyl PA (679), 1-myristoyl 2-oleoyl PA (645), 1-o-myristoyl 2-stearoyl PA (633), 1,2-sn-dilinoleoyl PA (695), 1-oleoyl 2-linoleoyl PA (697), 1-stearoyl 2-oleoyl PA (701), 1-o-oleoyl 2-20:4 PA (707), 1-o-linoleoyl 2-20:4 PA (705), 1-o-linoleoyl 2-20:5 PA (703), and combinations thereof.

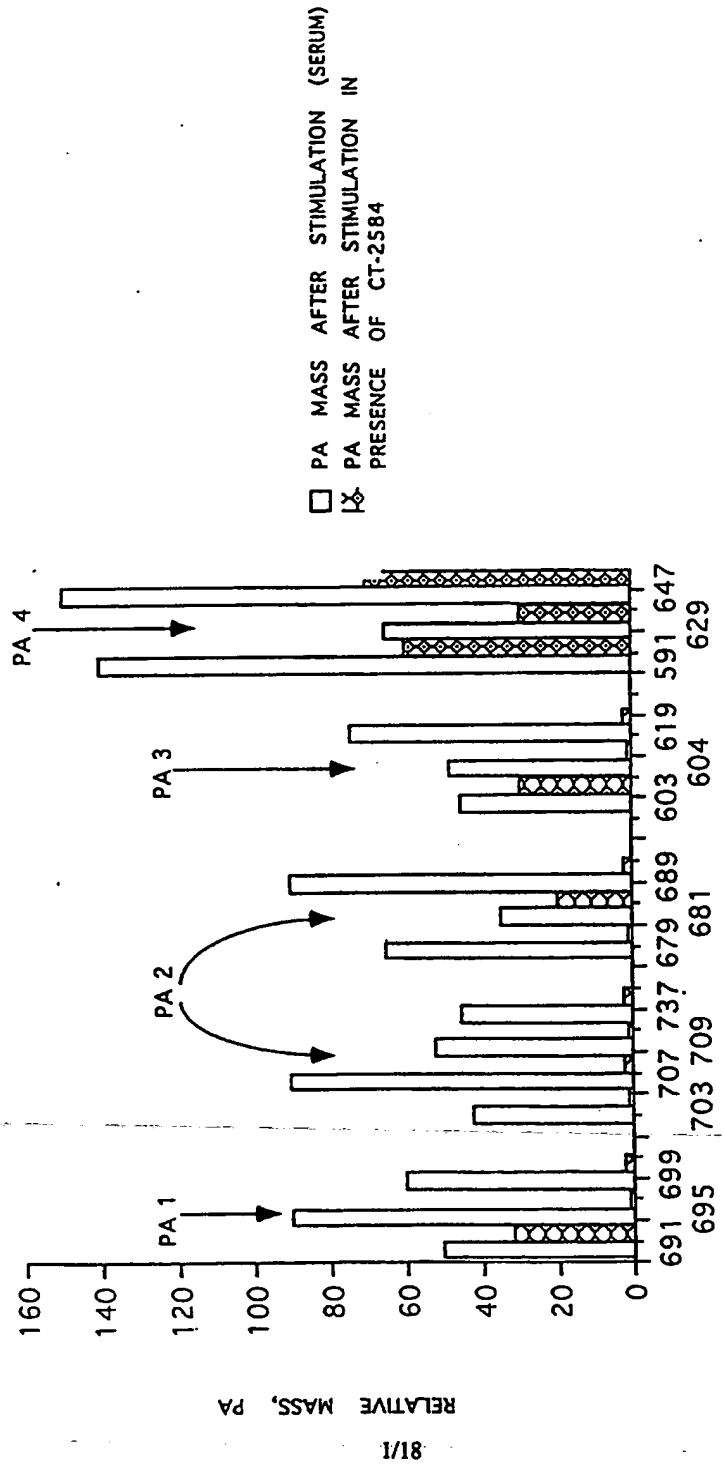

PA MASS AFTER STIMULATION (SERUM)
PA MASS AFTER STIMULATION IN PRESENCE OF CT-2584
RELATIVE MASS, PA
160
140
120
100
80
60
40
20
0
PA 1
PA 2
PA 3
PA 4
691 699 695
703 709 707 737 679 689 681
603 619 604
591 647 629

FIGURE 1

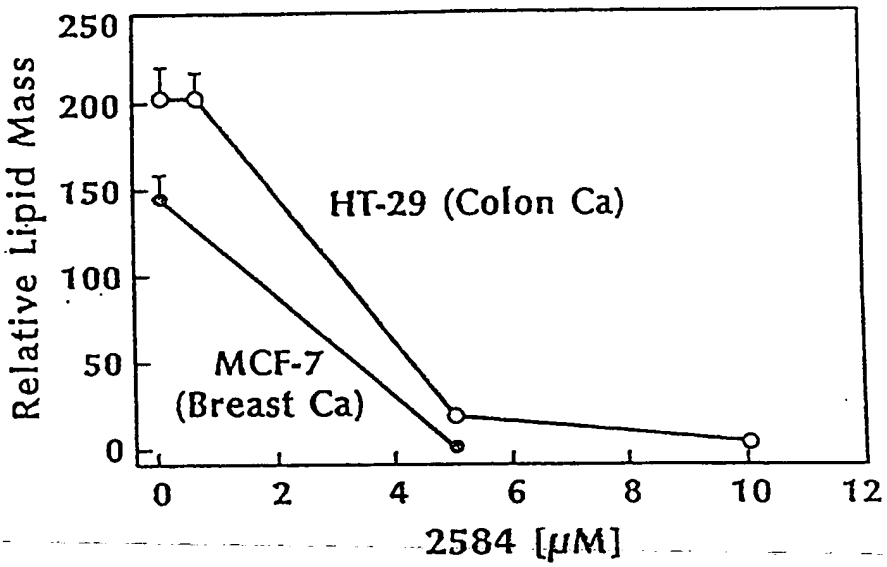

Relative Lipid Mass
250
200
150
100
50
0
HT-29 (Colon Ca)
MCF-7
(Breast Ca)
0
2
4
6
8
10
12
2584 [μM]

FIGURE 2

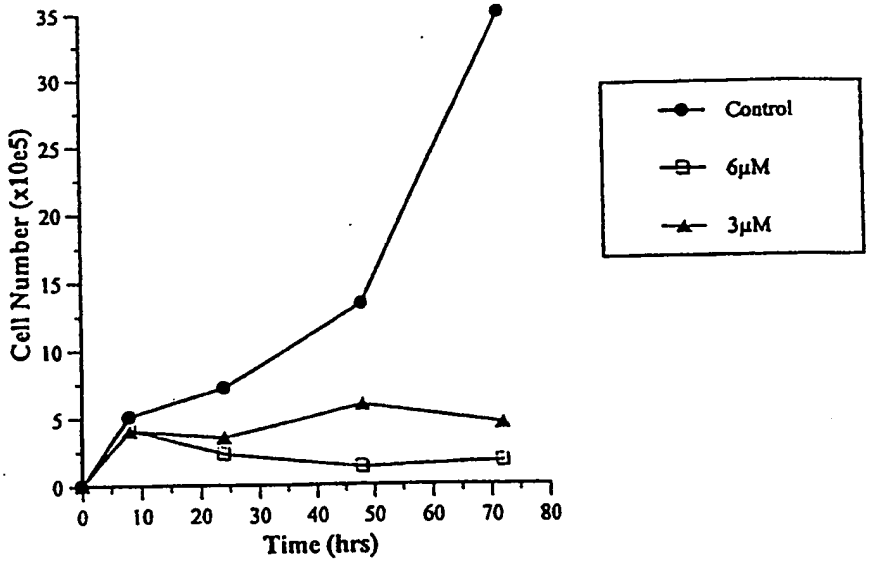


FIGURE 3

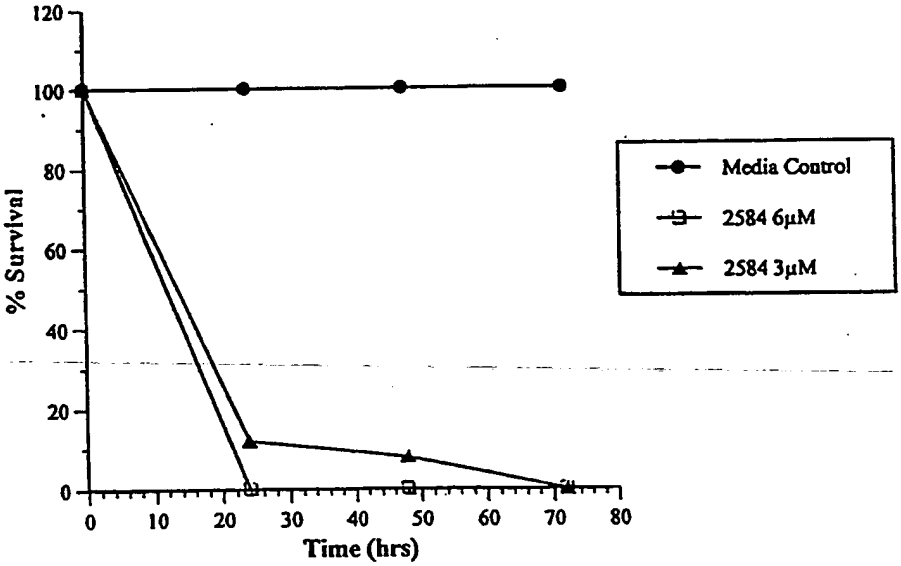


FIGURE 4

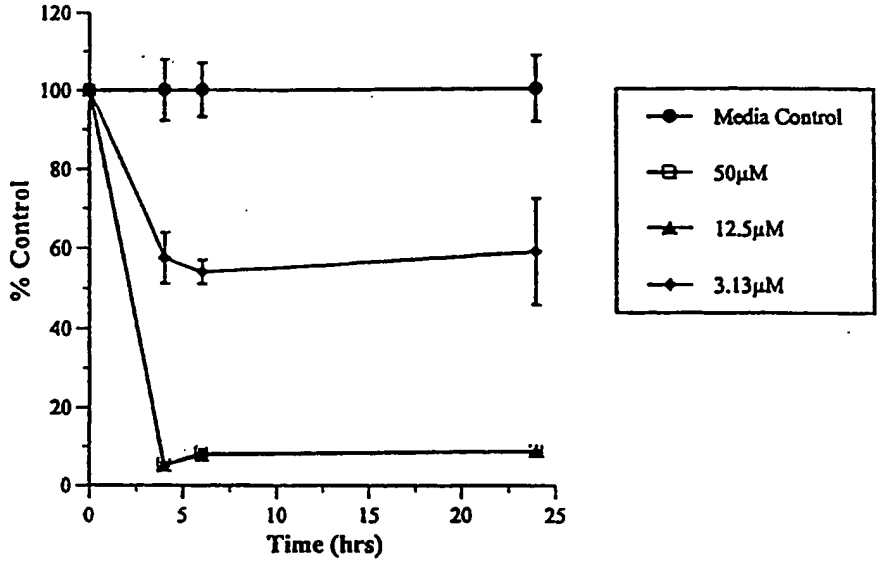


FIGURE 5

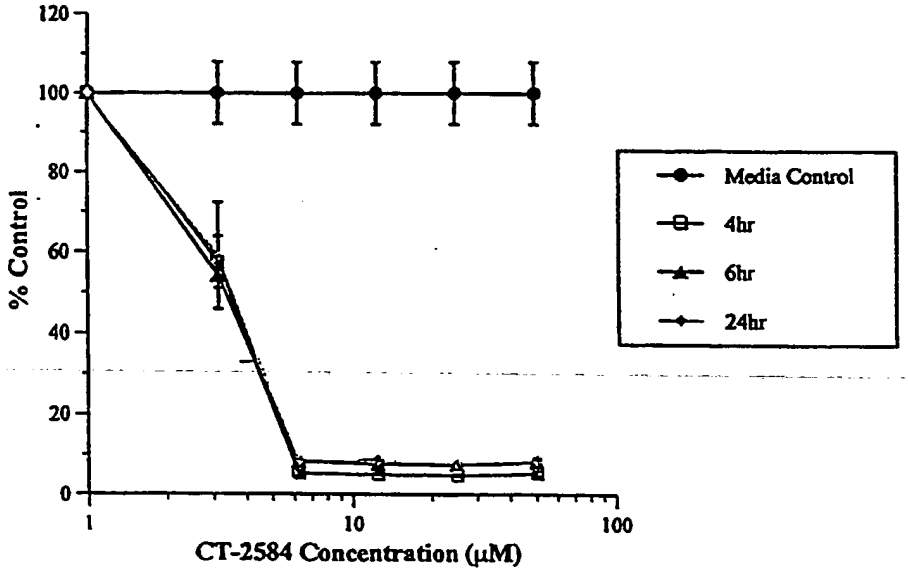


FIGURE 6

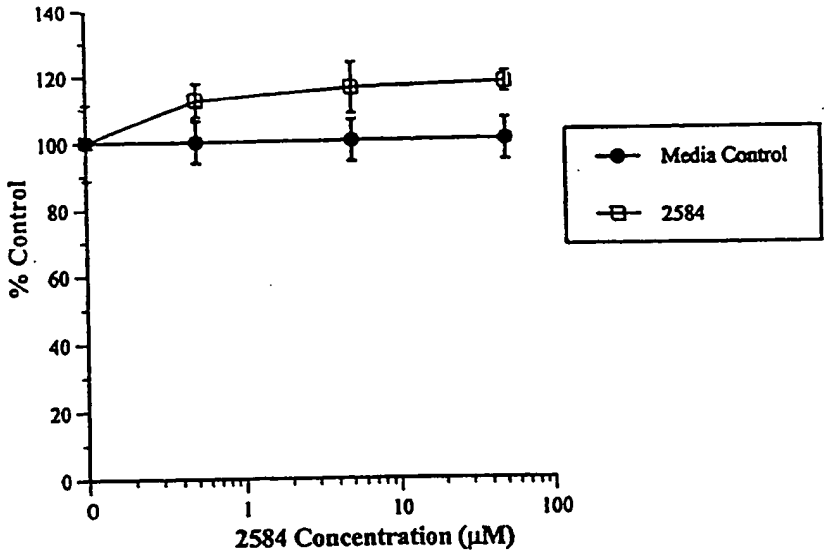

140
120
100
80
60
40
20
0
% Control
0
1
10
100
2584 Concentration (μM)
Media Control
2584

FIGURE 7

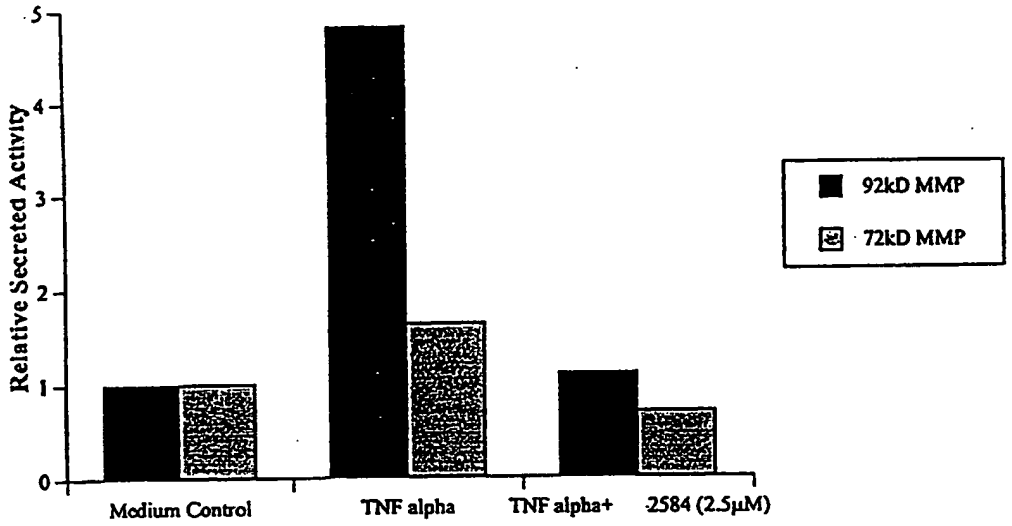

Relative Secreted Activity
0
1
2
3
4
5
Medium Control
TNF alpha
TNF alpha+
2584 (2.5μM)
92kD MMP
72kD MMP

FIGURE 8

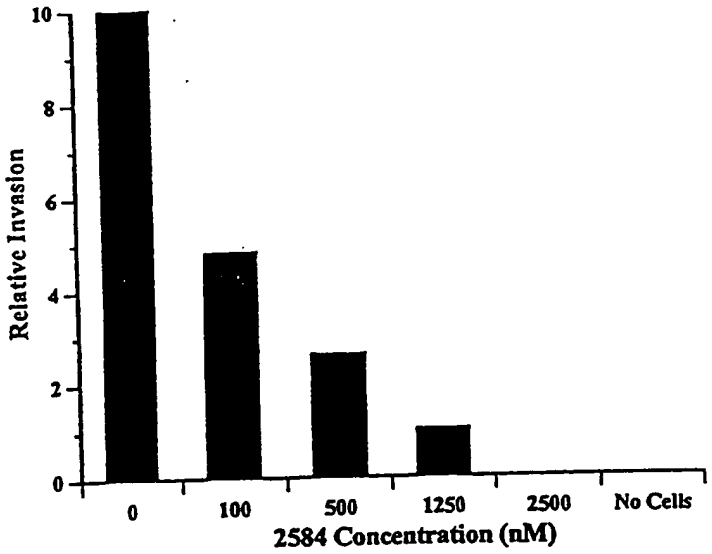


FIGURE 9

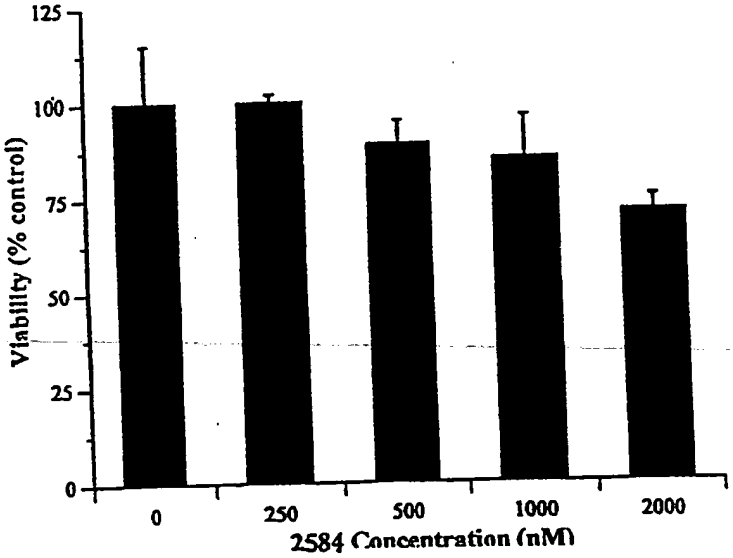


FIGURE 10

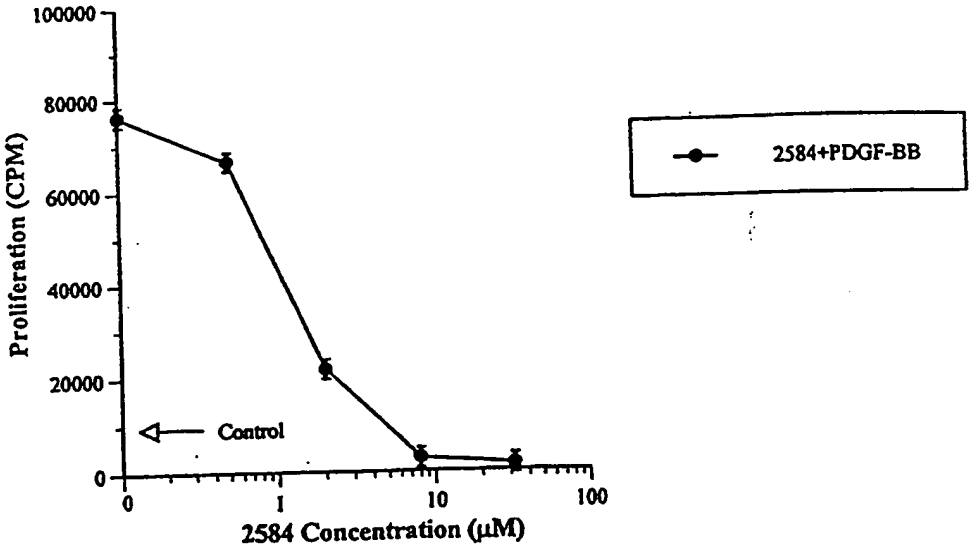

100000
80000
60000
40000
20000
0
Proliferation (CPM)
Control
0
1
10
100
2584 Concentration (μM)
2584+PDGF-BB

FIGURE 11

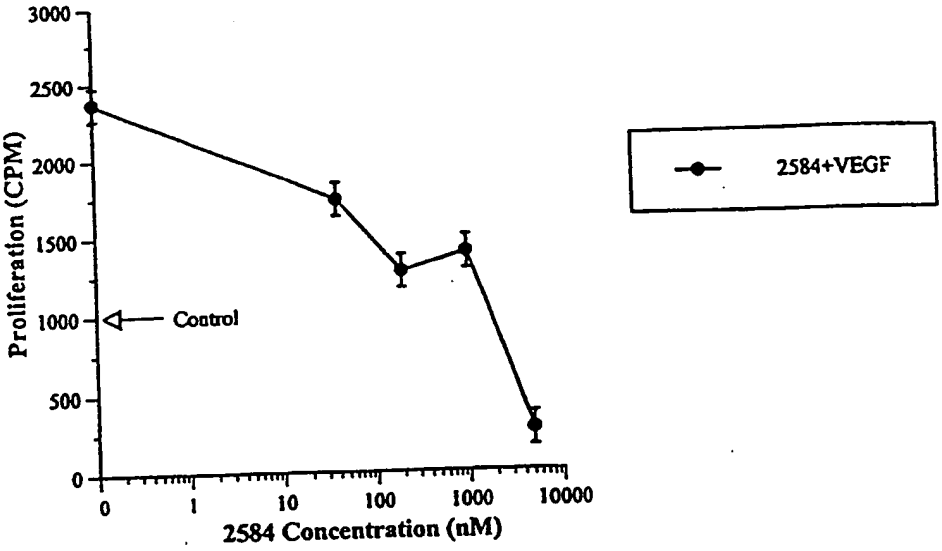

3000
2500
2000
1500
1000
500
0
Proliferation (CPM)
Control
0
1
10
100
1000
10000
2584 Concentration (nM)
2584+VEGF

FIGURE 12

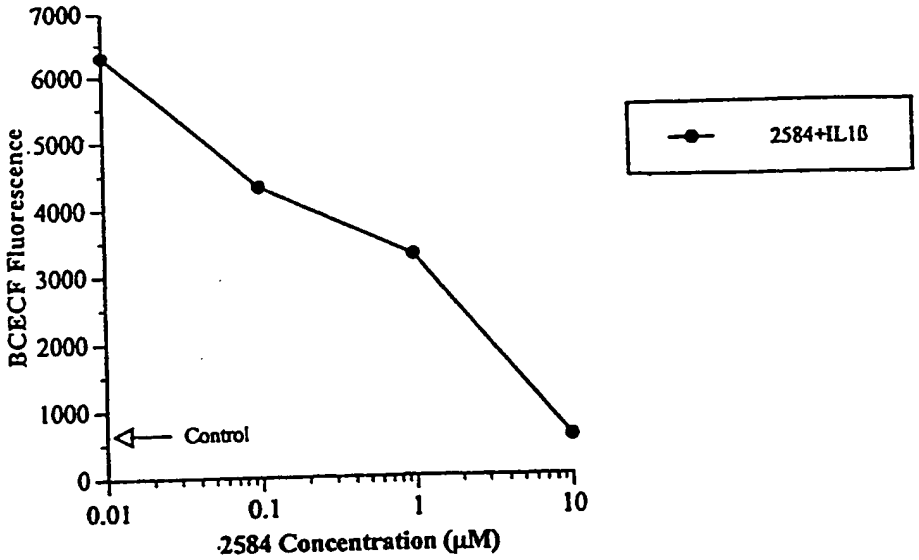

7000
6000
5000
4000
3000
2000
1000
0
BCECF Fluorescence
Control
0.01
0.1
1
10
2584 Concentration (μM)
2584+IL1β

FIGURE 13

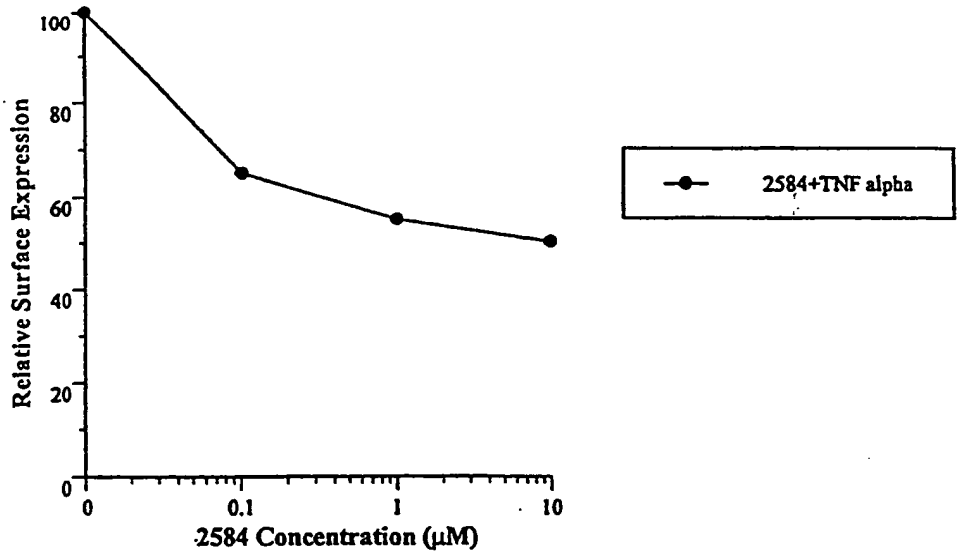

100
80
60
40
20
0
Relative Surface Expression
0
0.1
1
10
2584 Concentration (μM)
2584+TNF alpha

FIGURE 14

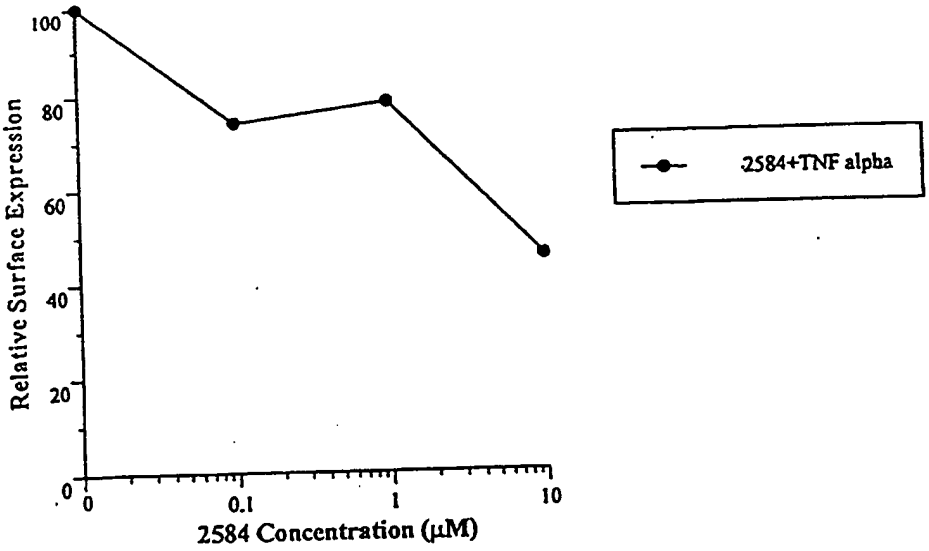

100
80
60
40
20
0
Relative Surface Expression
0
0.1
1
10
2584 Concentration (μM)
2584+TNF alpha

FIGURE 15

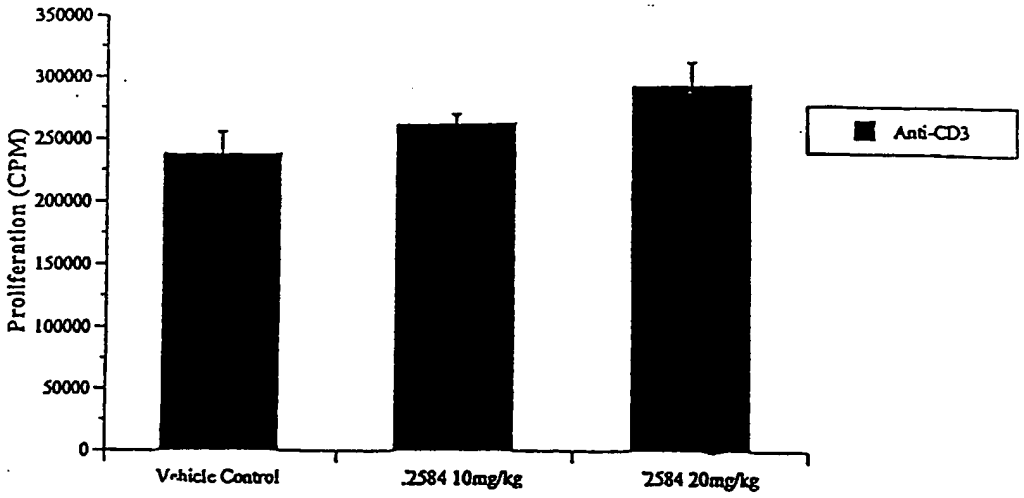


FIGURE 16A

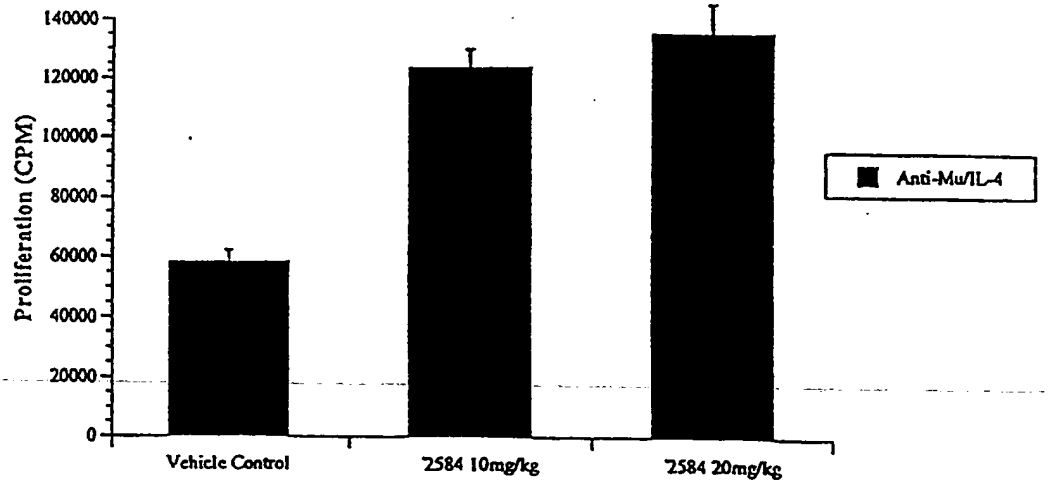


FIGURE 16B

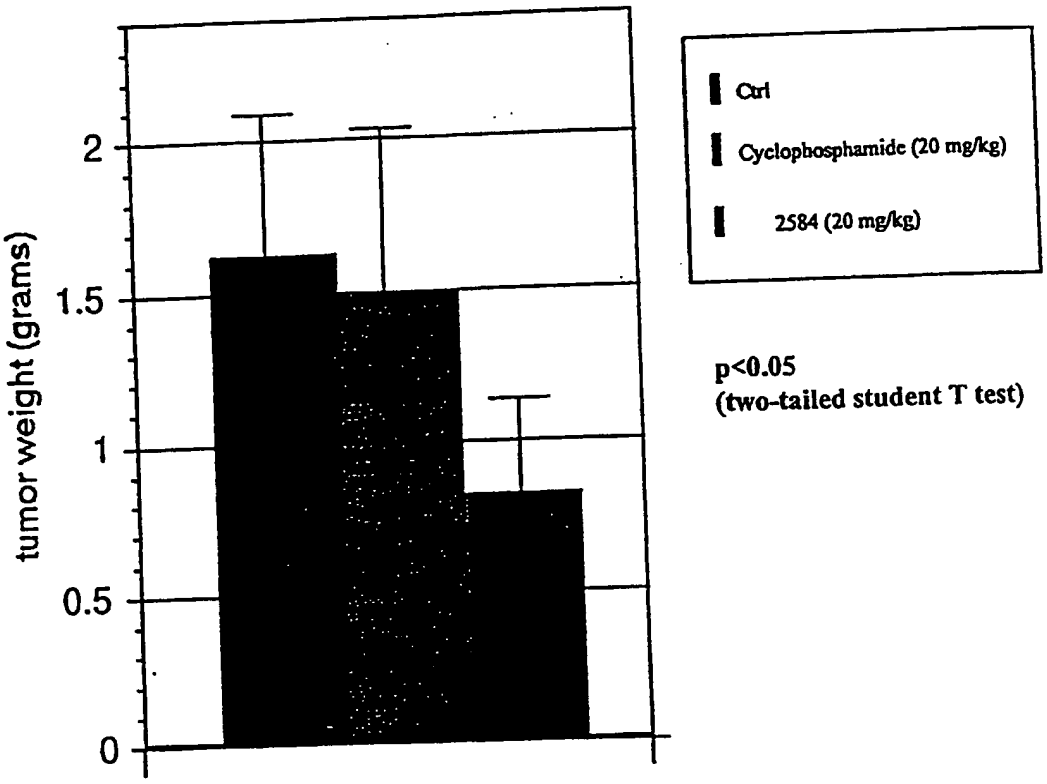

tumor weight (grams)
0
0.5
1
1.5
2
Ctrl
Cyclophosphamide (20 mg/kg)
2584 (20 mg/kg)
p<0.05
(two-tailed student T test)

FIGURE 17

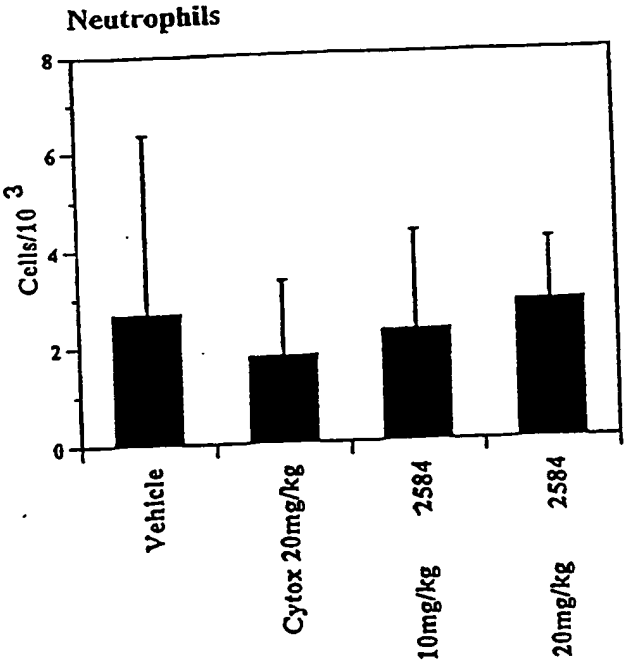


FIGURE 18A

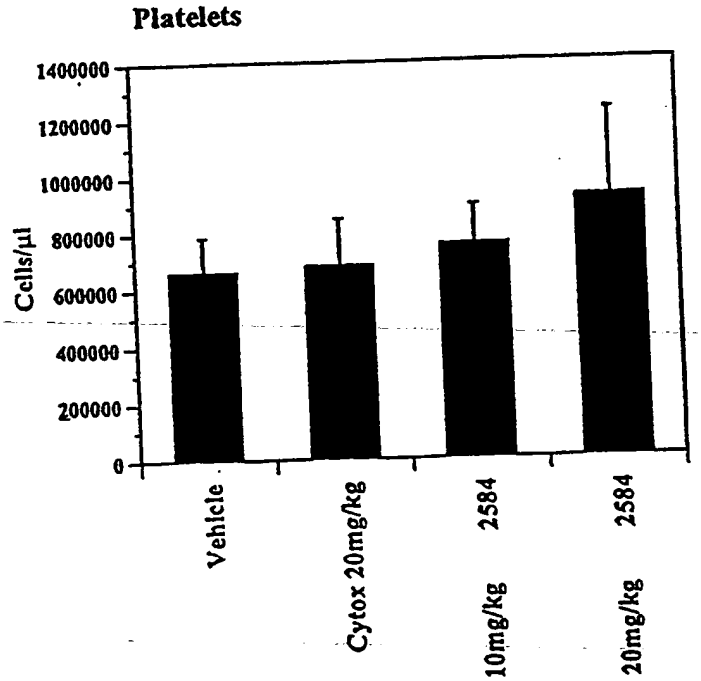


FIGURE 18B

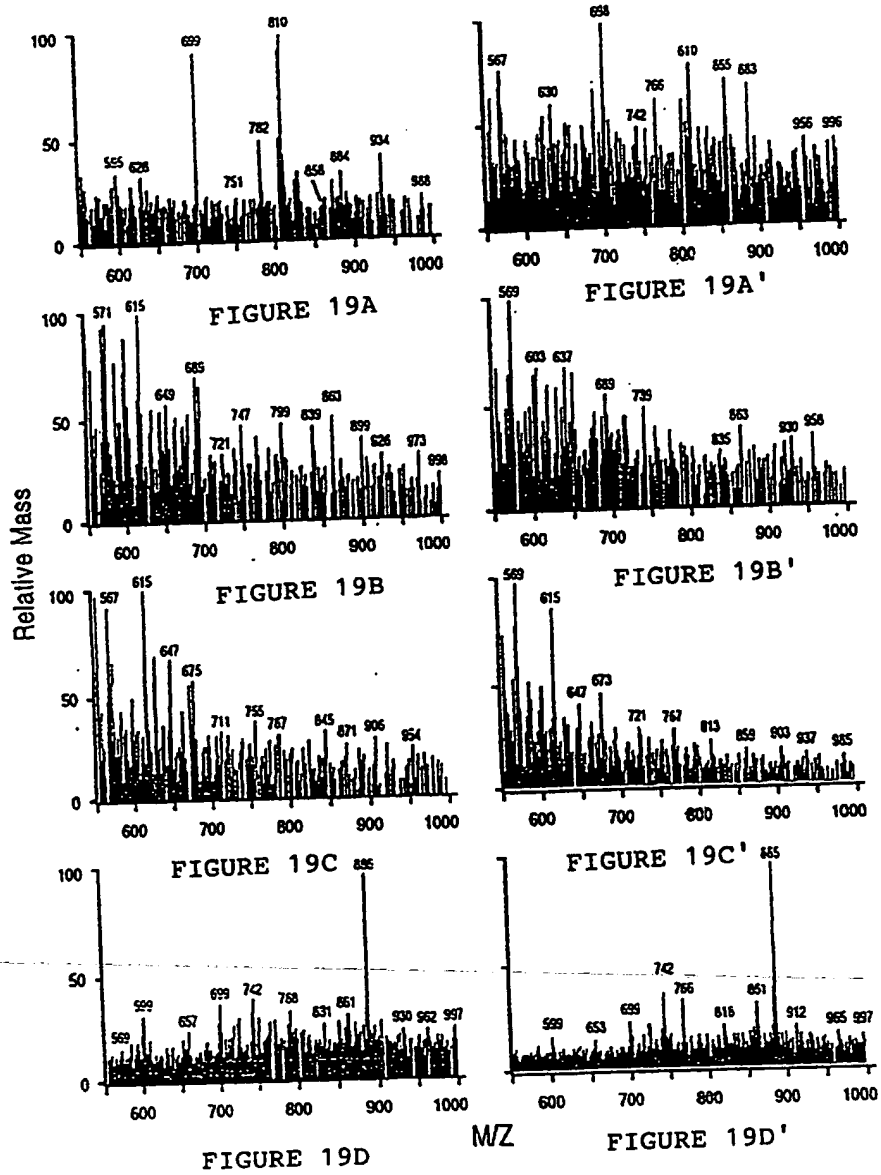


FIGURE 19A

FIGURE 19A'

FIGURE 19B

FIGURE 19B'

FIGURE 19C

FIGURE 19C'

FIGURE 19D

FIGURE 19D'

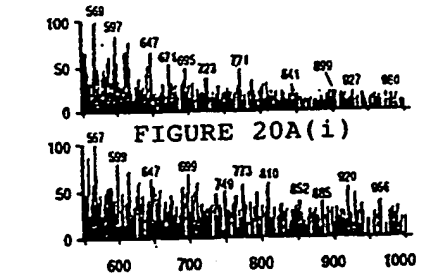


FIGURE 20A(i)

FIGURE 20A(ii)

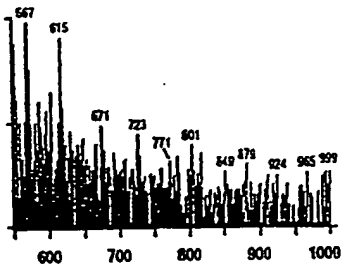


FIGURE 20A'

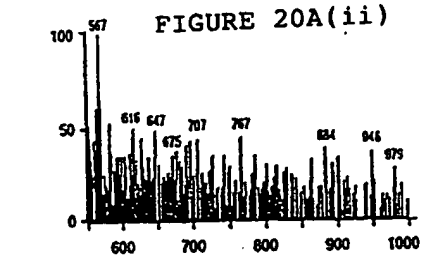


FIGURE 20B

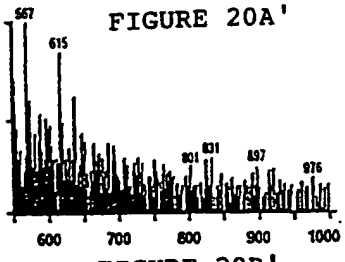


FIGURE 20B'

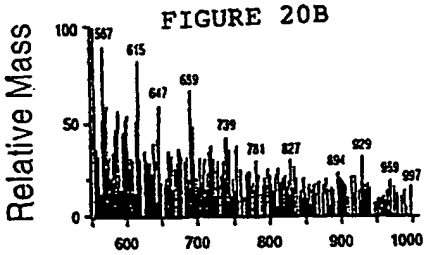


FIGURE 20C

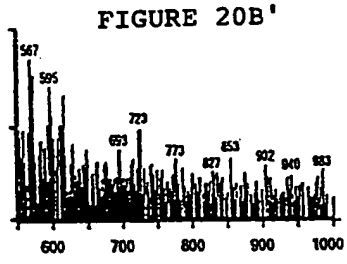


FIGURE 20C'

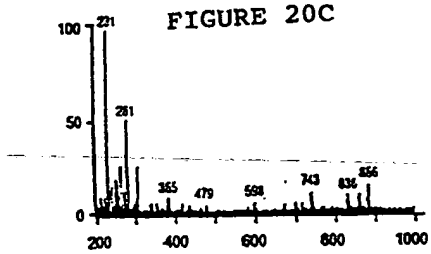


FIGURE 20D

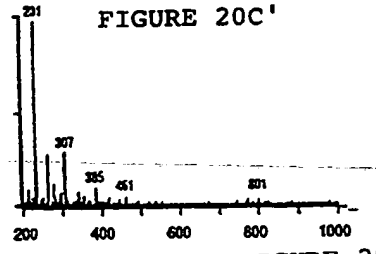


FIGURE 20D'

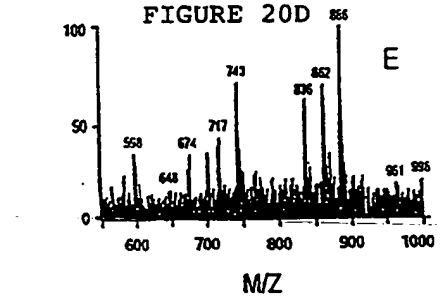


FIGURE 20E

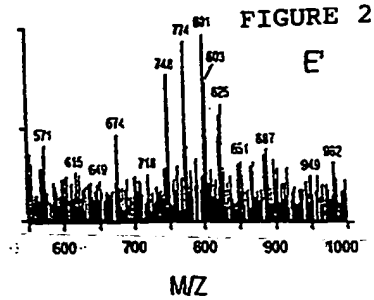
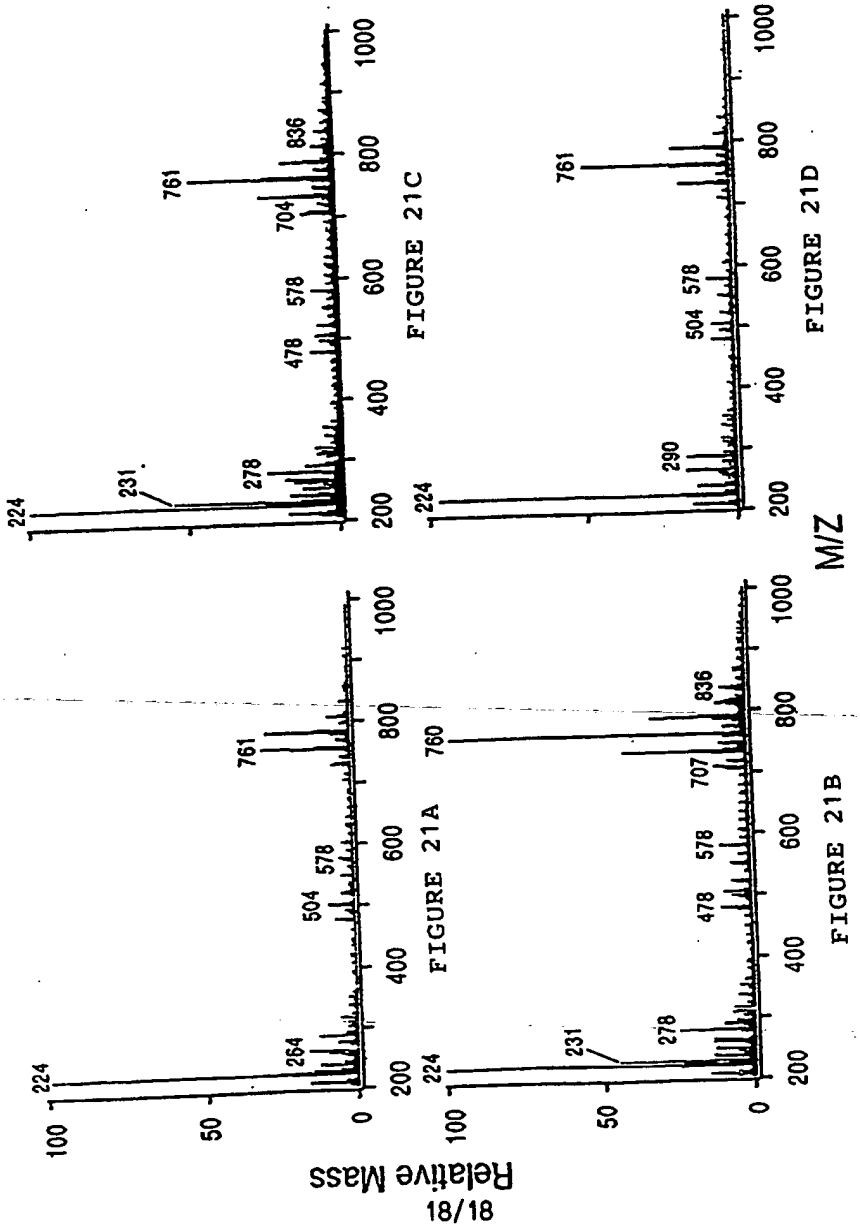


FIGURE 20E'


Relative Mass
M/Z
224
231
278
478
578
704
761
836
FIGURE 21C
224
290
504
578
761
FIGURE 21D
224
264
504
578
761
FIGURE 21A
224
231
278
478
578
707
760
836
FIGURE 21B

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(74) Agent: OSTER, Jeffrey, B.; Cell Therapeutics, Inc., Suite 400, 201 Elliot Avenue West, Seattle, WA 98119 (US).

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(57) Abstract

The present invention was made as a result of discovering an entire new approach and therapeutic target of cancer therapy. In addition, a prototype inhibitor compound, 1-(11-dodecylamino-10-hydroxyundecyl)-3,7-dimethylxanthine, has been discovered that acts by inhibiting cellular accumulation of specific PA species and is an inhibitor of PC directed PLDβ. Accordingly, the present invention provides a method for treating and preventing the spread of cancers comprising treating a patient with a tumor with an effective amount of a compound that reduces cellular accumulation of PA_4 species.

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CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

INTERNATIONAL SEARCH REPORT

Inte: ional Application No
PCT/US 95/03081

A. CLASSIFICATI N OF SUBJECT MATTER
IPC 6 A61K31/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,94 22863 (CELL THERAPEUTICS, INC.) 13 October 1994 see page 1, paragraph 1 see page 2, line 10 -paragraph 1 see page 9, line 24 - page 10, line 6 see page 12	1-8
X	US,A,5 288 721 (J.PETER KLEIN ET AL.) 22 February 1994 see abstract	1,7,8
Y	see column 2, line 58 - column 8, line 24	2,3,5,6

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 1995	27.10.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tzschoppe, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/03081

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 21344 (FRED HUTCHINSON CANCER RESEARCH CENTER) 10 December 1992	1,7
Y	see page 1, paragraph 2; claims 1-14 see page 3, line 15 - line 29 see page 6, paragraph 2 see page 9, line 12 - line 33 see page 19, line 15 - page 22, line 2	2,3,5,6
X	Cancerlit Abstract No. 95604126 & Proc. Annu. Meet.Am.Assoc. Cancer Res., vol. 35, A2441, March 1994 see abstract	1-4
X	Proc. Annu. Meet. Am.Assoc. Cancer Res., vol. 35, Abstract no. A401, March 1994	1-4
Y	see abstract	5,6
X	CIRCULATORY SHOCK, vol.44, no.1, pages 14 - 29 STUART BURSTEN ET AL. 'Potential role for phosphatidic acid in mediating inflammatory responses to TNF-alpha and IL-1beta'	1
Y	see abstract see page 15, right column, paragraph 2	2,3,5,6

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03081

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. claims 1-8
2. claim 9

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

claims 1-8

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Claims 1-6 and claim 9 relate both to the treatment of cancer. However the mechanism by which this treatment is effected is completely different according to claims 1-6 and claim 9 respectively. These different mechanisms cannot be considered as forming a common inventive concept. The only common feature which could form a common inventive concept linking the subject-matter of claims 1-6 and the subject-matter of claim 9 is the treatment of cancer. This is however already known for the compounds inhibiting the formation of phosphatidic acid (see documents cited in the search report), so that this common concept is not novel.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 95/03081

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9422863	13-10-94	AU-B-	6553894	24-10-94
US-A-5288721	22-02-94	AU-B-	5138493	12-04-94
		CA-A-	2145192	31-03-94
		EP-A-	0662834	19-07-95
		WO-A-	9406431	31-03-94
WO-A-9221344	10-12-92	AU-A-	2247592	08-01-93
		EP-A-	0573617	15-12-93
		JP-T-	7500085	05-01-95